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Environment

Titanium dioxide nanoparticles promote histopathological and genotoxic effects in *Danio rerio* after acute and chronic exposures

Nanopartículas de dióxido de titânio promovem efeitos histopatológicos e genotóxicos em *Danio rerio* após exposição aguda e crônica

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2-NPs) are among the most used nanomaterials worldwide, but studies evaluating its genotoxicity and histopathological effects are scarce, dealing with short exposure times and low concentrations for human use. The aim was to evaluate TiO_2-NPs genotoxicity and histological alterations in the intestine and liver of zebrafish after exposure to human consumption compatible concentrations. Fishes were acutely (96 hours) and chronically (30 days) exposed to 5.0, 20 and 40 mg L^{-1} of TiO_2-NPs and later euthanized for organ and blood analysis through histological procedures and the micronucleus test, respectively. An increase in the thickness of intestinal villi was observed after acute and chronic exposure in the higher concentrations. The liver showed an increase in vacuolated hepatocytes after both exposures, besides an increase in hepatocytes with peripheral nucleus. Genotoxicity was only observed after chronic exposure, demonstrated by the increase in micronucleus and cell buddings. These findings indicate that TiO_2-NPs cause histopathological damage even in acute exposures, as the intestine serves as a barrier for NPs and the liver is an organ that accumulates Ti. Genotoxicity was possibly mediated by reactive oxygen species through chronic inflammation, leading to tissue damage and carcinogenesis in longer exposures that represents human exposure time.

Keywords: Intestine; Liver; Micronucleus test; Nanotoxicology; Zebrafish

RESUMO

Nanopartículas de dióxido de titânio (Nano-TiO_2) estão entre os nanomateriais mais usados em todo o mundo, mas os estudos avaliando sua genotoxicidade e efeitos histopatológicos são escassos, tratando-se de curtos tempos de exposição e baixas concentrações para uso humano. O objetivo foi avaliar a genotoxicidade de Nano-TiO_2 e as alterações histológicas no intestino e no fígado de peixes-zebra após exposição a concentrações compatíveis com o consumo humano. Os peixes foram expostos agudamente (96 horas) e cronicamente (30 dias) a 5, 20 e 40 mg L^{-1} de Nano-TiO_2 e posteriormente eutanasiados para análise de órgãos e sangue por meio de procedimentos histológicos e teste de micronúcleo, respectivamente. Um aumento na espessura das vilosidades intestinais foi observado após exposição aguda e crônica nas concentrações mais altas. O fígado apresentou aumento de hepatócitos vacuolizados após ambas as exposições, além de aumento de hepatócitos com núcleo periférico. A genotoxicidade só foi observada após exposição crônica, demonstrada pelo aumento de micronúcleos e brotamentos celulares. Esses achados indicam que as Nano-TiO_2 causam danos histopatológicos mesmo em exposições agudas, pois o intestino serve como uma barreira para as NPs e o fígado é um órgão que acumula Ti. A genotoxicidade foi possivelmente mediada por espécies reativas de oxigênio por meio de inflamação crônica, levando a danos nos tecidos e carcinogênese em exposições mais longas que representam o tempo de exposição humana.

Palavras-chave: Intestino; Fígado; Teste de micronúcleo; Nanotoxicologia; Peixe-zebra

1 INTRODUCTION

Nanotechnology has been widely explored in different spheres of human activities and it is already incorporated in commercial products and industrial processes (ASZTEMBORSKA *et al*., 2018; THOMAS *et al*., 2011). Nanomanufacturing provides the improvement and development of new food, drugs, electronics, cosmetics, agricultural and medical-hospital inputs with unique physicochemical characteristics (CHEN; SCHLUESENER, 2008; TANG; ZHANG; ZHU, 2019). However, the increase in nanomaterial production began to be questioned because of the possible effects of human exposure and environmental risk (HAYES; SAHU, 2020).

Titanium dioxide (TiO_2) is a mineral that has high thermal stability, resistance to color change, and anti-corrosion and photocatalytic characteristics (KOTIL; AKBULUT; YÖN, 2017). These properties turn TiO_2 useful for: toothbrushes, cosmetics, paints, glasses, paper, plastics, sunscreen, food, and pharmaceuticals (DUDEFOI *et al*., 2017; KOTIL; AKBULUT; YÖN, 2017). To improve the effectiveness of these products, TiO_2 has been increasingly produced in nanometric dimensions, representing the most produced nanomaterial worldwide (PICCINNO *et al*., 2012;

ROBICHAUD *et al*., 2009). In the manufacturing process, titanium dioxide nanoparticles (TiO_2-NPs) can be presented as agglomerates or aggregates greater than 100 nm, resulting changes in properties, such as transport and reactivity (BOVERHOF *et al*., 2015; MAURICE; HOCHELLA, 2008). In fact, it is the increase in reactivity that can contribute to the expansion of toxicity, which is why the use of TiO_2-NPs is still controversial (KHAN *et al*., 2019).

As a food additive, TiO_2 (E171) use is regulated in Brazil and other countries (ANVISA, 2018; EFSA, 2016; EP, 2015; FDA, 2020) in two chemical forms, anatase and rutile, that are applied in a broad range of foods, such as cheese, sauces, skim milk, ice cream, sweets, confectionery, and chewing gum (EFSA, 2016; WEIR *et al*., 2012; YIN *et al*., 2017). In addition, human oral exposure to TiO_2 can also unintentionally occur through contaminated water (BOUWMEESTER; ZANDE; JEPSON, 2017). TiO_2-NPs ingestion is related to elevated intestinal absorption and bioavailability that affects the distribution process and tissue retention (AMMENDOLIA *et al*., 2017). In order to clarify these mechanisms, studies have been performed to evaluate TiO_2-NPs toxicity in different animal models (CHEN *et al*., 2019; ZHU *et al*., 2010).

The aquatic biota is exposed to several contaminants in water (DALZOCHIO *et al*., 2018). Considered excellent bioindicators, fishes are also susceptible to agents located in food and sediment like TiO_2-NPs, that has water its final destiny and can easily move from the water column to the aquatic food chain (BOXALL; TIEDE; CHAUDHRY, 2007; ZHU *et al*., 2010). When in contact with nanoparticles, these animals tend to absorb them through the gills, in the breathing process, and through the intestine, from the diet (CARMO *et al*., 2018). Previous studies have shown that acute exposure to TiO_2-NPs is sufficient for titanium (Ti) bioaccumulation in *Prochilodus lineatus*, while chronic exposures can cause oxidative damage in *Danio rerio* (CARMO *et al*., 2019; TANG; ZHANG; ZHU, 2019). The latter species, popularly known as zebrafish, is widely used in biomedical and toxicological research, and also has several orthologous genes to humans and presents similar cellular types to mammals in the cardiovascular, nervous, and

digestive systems (HSU *et al*., 2007; SIPES; PADILLA; KNUDSEN, 2011). However, studies evaluating TiO_2-NPs genotoxicity in *Danio rerio* are extremely scarce, dealing with relatively low concentrations found in effluents, in the range of μg L^{-1}, and acute exposure times that make it difficult to observe long-term effects (BOBORI *et al*., 2020; ROCCO *et al*., 2015). There is also limited information about histopathological alterations caused by TiO_2-NPs exposure in *Danio rerio*, although TiO_2 has been already nominated as a possible carcinogenic to humans (B2) based on animal studies (IARC, 2010).

Therefore, the aim of this study was to evaluate TiO_2-NPs genotoxicity and histological alterations in the intestine and liver in *Danio rerio* after acute and chronic exposure to human consumption compatible concentrations.

2 MATERIAL AND METHODS

2.1 Preparation and characterization of TiO_2-NPs

TiO_2 in the anatase (Brenntag Química Brasil Ltda., purity ≥ 99%) and rutile (GuoBen [Shanghai] Enterprise Development Co. Ltd., purity ≥ 92%) phases were obtained from local industries. A mixture of 75% anatase and 25% rutile was prepared for nanoparticle characterization and use in the experiments, according to previous studies with fish (BAR-ILAN *et al*., 2012; FEDERICI; SHAW; HANDY, 2007; ROCCO *et al*., 2015). TiO_2-NPs characterization was performed by two different techniques: 1) scanning electron microscopy (SEM) coupled to X-ray energy dispersive spectrometry (EDS) and 2) dynamic light scattering (DLS).

In order to assess the morphology and size of the primary particles, micrographs of anatase, rutile and mixture were taken at a x 40,000 magnification in a scanning electron microscope (SEM; Jeol JAM-6610LV) operating at 29 kV. The elemental chemical composition was determined at a x 10,000 magnification (29 kV) by EDS using a light elements detector (Thermo Fisher Scientific UltraDry).

DLS technique was applied to determine the particle size distribution and the polydispersity index of the nanomaterials. Three suspensions (anatase, rutile and mixture) were prepared in N,N-dimethylformamide (2 mg mL^{-1}; Êxodo Científica®), sonicated for 10 min (Ultronique, disruptor 500W model) and centrifuged for 10 min at 3000 rpm. The measurements were performed in triplicate (5 minutes each) in the NanoBrook 90 PlusPals® (Brookhaven Instruments) at 25 ºC and a 90° scattering angle. The software used for analysis and visualization of DLS data was the Particle Solutions® v. 3.5 (Brookhaven Instruments). Particle diameter (nm) and polydispersity index (PdI) were expressed as mean ± standard error.

For the experiments, a stock solution of the mixture (75% anatase and 25% rutile) was prepared in reconstituted water (ISO, 1996) and kept refrigerated. The same stock solution was used to obtain the concentrations (5.0, 20 and 40 mg L^{-1}) during the entire experimental period. The concentrations were defined based on the estimated daily exposure to TiO_2 as a food additive in adults (0.6 to 6.8 mg kg^{-1} per day), considering the hypothesis that an adult who has a body mass of 70 kg and is minimally exposed has a daily consumption of 42 mg of TiO_2 (EFSA, 2016).

2.2 Experimental procedures

Procedures described below were previously approved by the Institutional Committee for Animal Care and Use from Feevale University (03.19.079), according to the Law 11794/2008. Adult wild-type zebrafish (*Danio rerio*; 6-8 months old, males and females) were obtained from a local supplier. In the laboratory, animals were acclimatized (30 days) in reconstituted water in the same environmental conditions of experimentation: water temperature of 26 ± 2 ºC, pH at 7.0 – 8.0, water hardness between 75 and 100 mg L^{-1} $CaCo_3$, and a maximum density of two animals per liter of water (ABNT, 2016). The light/dark cycle was 14:10 h (lights on at 7:00 a.m.), the water of the aquariums was in constant aeration (semi-static

system), and the animals were fed thrice per day: twice with commercial flake food (Tetra®) and once with live brine shrimp (*Artemia sp*.).

This study consisted of two exposure periods, acute (96 hours) and chronic (30 days), with 48 animals each. The animals of both periods (12 animals per group) were exposed to 5.0, 20 and 40 mg L^{-1} of TiO_2-NPs, and the control group was kept in reconstituted water. In both experiments, 100% of the water was renewed every 48 hours due to the tendency of particle aggregation and sedimentation. Fish survival was assessed during all experiments.

2.3 Turbidity analysis

Three water samples (50 mL), one of each concentration, were collected from the middle region of the aquariums in the acute and chronic exposure. The analysis was performed in triplicate in a Hach® 2100N turbidimeter and the equipment was properly calibrated with turbidity standards (StablCal®). The samples were homogenized by agitation and the values were obtained in NTU (Nephelometric Turbidity Unit) after 60 seconds of measurement. Data were expressed as mean ± standard error. None of the samples were diluted or modified before turbidity measurements to represent the real ambient condition. The analysis was performed every 48 hours during the acute experiment and weekly in the chronic experiment. The samples were always collected from the aquariums before water renovation.

2.4 Histological procedures

Animals were anesthetized and euthanized with tricaine methanesulfonate (MS-222; Sigma-Aldrich®, 120 mg L^{-1}). The intestine and liver from half of the animals of each group (n = 6) were removed and fixed in 10% formaldehyde (LabSynth®). Subsequently, the samples were dehydrated in a gradual series of ethanol, embedded in paraffin, sectioned (5 µm) (Leica® RM2125RT) and stained

with hematoxylin and eosin (H&E) for liver analysis or with Alcian blue and hematoxylin for intestine analysis. Both were performed through coded photomicrographs captured with an optical microscope (Nikon Eclipse E200) coupled to a camera (Precision) at a x 400 magnification.

For the liver analysis, we adapted the classification adopted by Rodrigues *et al*. (2020), accounting the number of normal hepatocytes, cells with vacuolization, and hepatocytes with a peripheral nucleus in 5 fields per animal. Regarding the intestine analysis, 5 fields per animal were analyzed by evaluating the percentage of altered villi in each field (2 villi per field). The increase in the thickness of intestinal villi, leukocyte infiltration, and presence/absence of eosinophils were registered according to the classification used by Rodrigues *et al*. (2018). The percentage of cellular alterations was calculated, and the number of intestinal goblet cells was also counted. All histological data were expressed as mean ± standard error.

2.5 Micronucleus test and nuclear abnormalities

Blood smears, obtained through the caudal section (6 animals per group), were fixed in methanol (10 minutes) and stained with Giemsa 5% solution (New Prov) for 10 minutes. The analysis was performed using light microscopy (Nikon Eclipse E100) at a x 1000 magnification. A total of 3000 erythrocytes were counted per fish, recording the number of cells with micronuclei and nuclear abnormalities (cellular invagination, binucleated cells, and cell budding) (FENECH *et al*., 2003; RODRIGUES *et al*., 2020). Data were expressed as mean ± standard deviation for micronuclei and mean ± standard error for nuclear abnormalities.

2.6 Statistical analysis

The data normality was assessed by the Kolmogorov-Smirnov test and $p < 0.05$ was considered significant. For micronucleus test, the Kruskal-Wallis test was

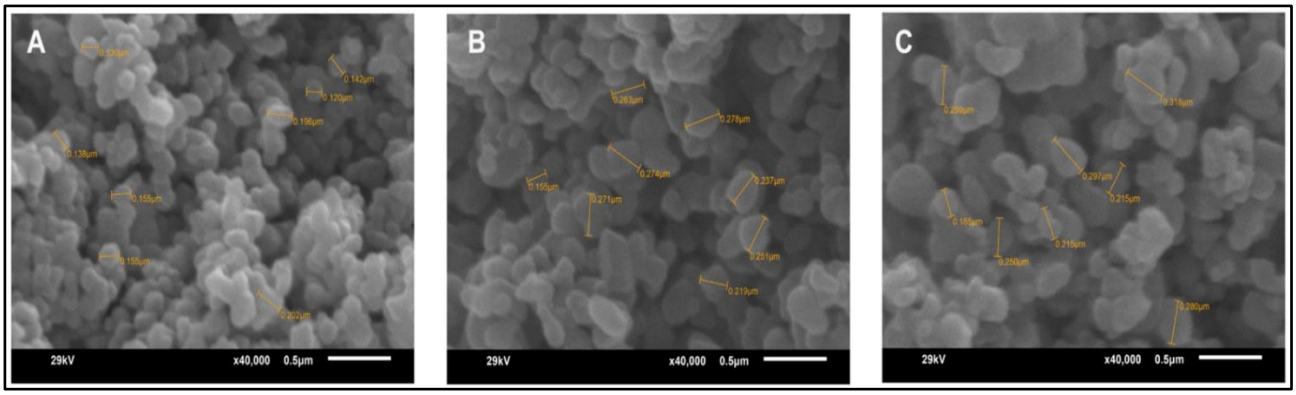
used, followed by the Dunn post-test. For nuclear abnormalities and histological analyses, the one-way Anova test was applied, followed by the Tukey post-hoc. For genotoxicity test, the comparison between acute and chronic exposure was performed using the Mann-Whitney test, while the Student's t-test was applied for histological analyses. The software used for all statistics was GraphPad Prism v. 6.0.

3 RESULTS

3.1 Characterization

The SEM images (Figure 1) show the presence of a similar spherical shape in both phases, and agglomerated titanium dioxide nanoparticles within a size range of 100 and 400 nm. In comparison to rutile nanoparticles, anatase seems to have smaller particles than rutile.

Figure 1 – Scanning electron microscopy images of titanium dioxide nanoparticles



Source: Authors' private collection (2021)
Caption: (A) Anatase TiO_2-NPs (B) Rutile TiO_2-NPs (C) Mixture TiO_2-NPs (75/25 anatase/rutile). The bars (|---|) indicate an estimated diameter (μm) of the particles.

The diameter and the polydispersity index of the prepared suspensions are presented in Table 1. As seen in SEM, TiO_2 has a huge tendency to aggregate and agglomerate, but the N,N-dimethylformamide was capable of stabilizing

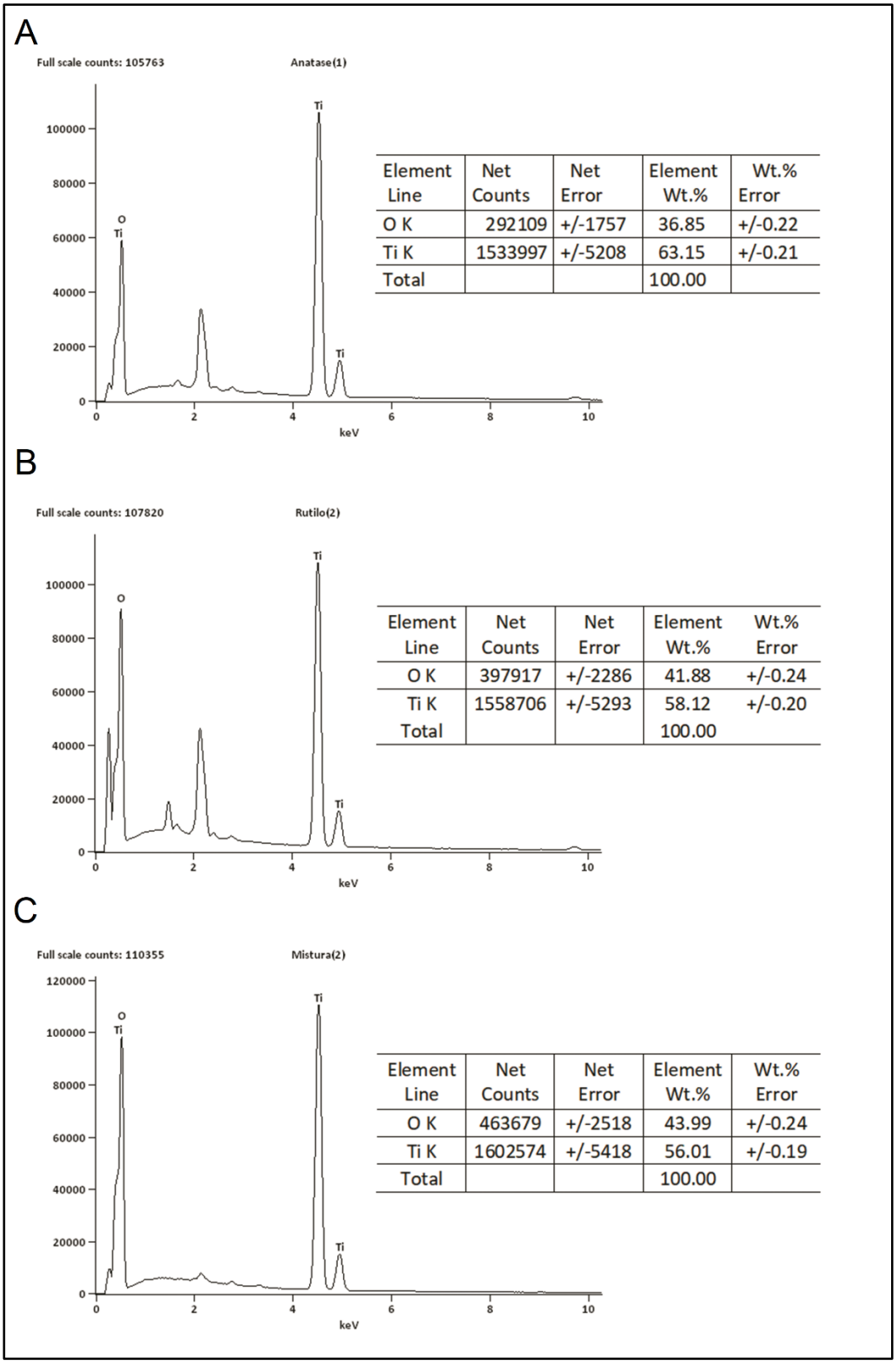
nanoparticles in the suspension. The diameters measured by DLS confirm that rutile has bigger particles than anatase, although the mixture diameter is not much affected. In addition, the polydispersity index shows that all the samples were monodispersed. The EDS analysis (Figure 2) demonstrated that the agglomerates seen in SEM images were made up of titanium and oxygen.

Table 1 – Particle diameter and polydispersity index of TiO_2-NPs in N, N-dimethylformamide (2 mg mL^{-1}) obtained by the DLS analysis

Parameter	**TiO_2-NPs phase**		**Mixture 75/25 anatase/rutile**
	Anatase	Rutile	
Particle diameter (nm)	190.32 ± 1.01	248.36 ± 0.46	189.64 ± 2.19
Polydispersity index (PdI)	0.124 ± 0.003	0.169 ± 0.002	0.154 ± 0.019

Source: Authors' private collection (2021)
Caption: Data are expressed as mean ± standard error. TiO2-NPs: titanium dioxide nanoparticles.

Figure 2 – EDS spectrum of titanium dioxide nanoparticles



Element Line	Net Counts	Net Error	Element Wt.%	Wt.% Error
O K	292109	+/-1757	36.85	+/-0.22
Ti K	1533997	+/-5208	63.15	+/-0.21
Total			100.00	

Element Line	Net Counts	Net Error	Element Wt.%	Wt.% Error
O K	397917	+/-2286	41.88	+/-0.24
Ti K	1558706	+/-5293	58.12	+/-0.20
Total			100.00	

Element Line	Net Counts	Net Error	Element Wt.%	Wt.% Error
O K	463679	+/-2518	43.99	+/-0.24
Ti K	1602574	+/-5418	56.01	+/-0.19
Total			100.00	

Source: Authors' private collection (2021)
Caption: A) Anatase TiO_2-NPs (B) Rutile TiO_2-NPs (C) Mixture TiO_2-NPs (75/25 anatase/rutile).

3.2 Turbidity of aquarium water samples

Turbidity data of aquarium water samples collected during the acute and chronic experiments are presented in Table 2. In both exposure times, the turbidity was directly proportional to TiO_2-NPs concentrations.

Table 2 – Turbidity of the aquarium water samples during the acute (96 hours) and chronic (30 days) experiments of the three concentrations of TiO_2-NPs

TiO_2-NPs concentration	**Turbidity (NTU)**	
	Acute exposure	Chronic exposure
5.0 mg L^{-1}	22.86 ± 2.36	57.64 ± 2.09
20 mg L^{-1}	49.56 ± 9.52	202.66 ± 4.61
40 mg L^{-1}	82.94 ± 21.35	376.40 ± 16.37

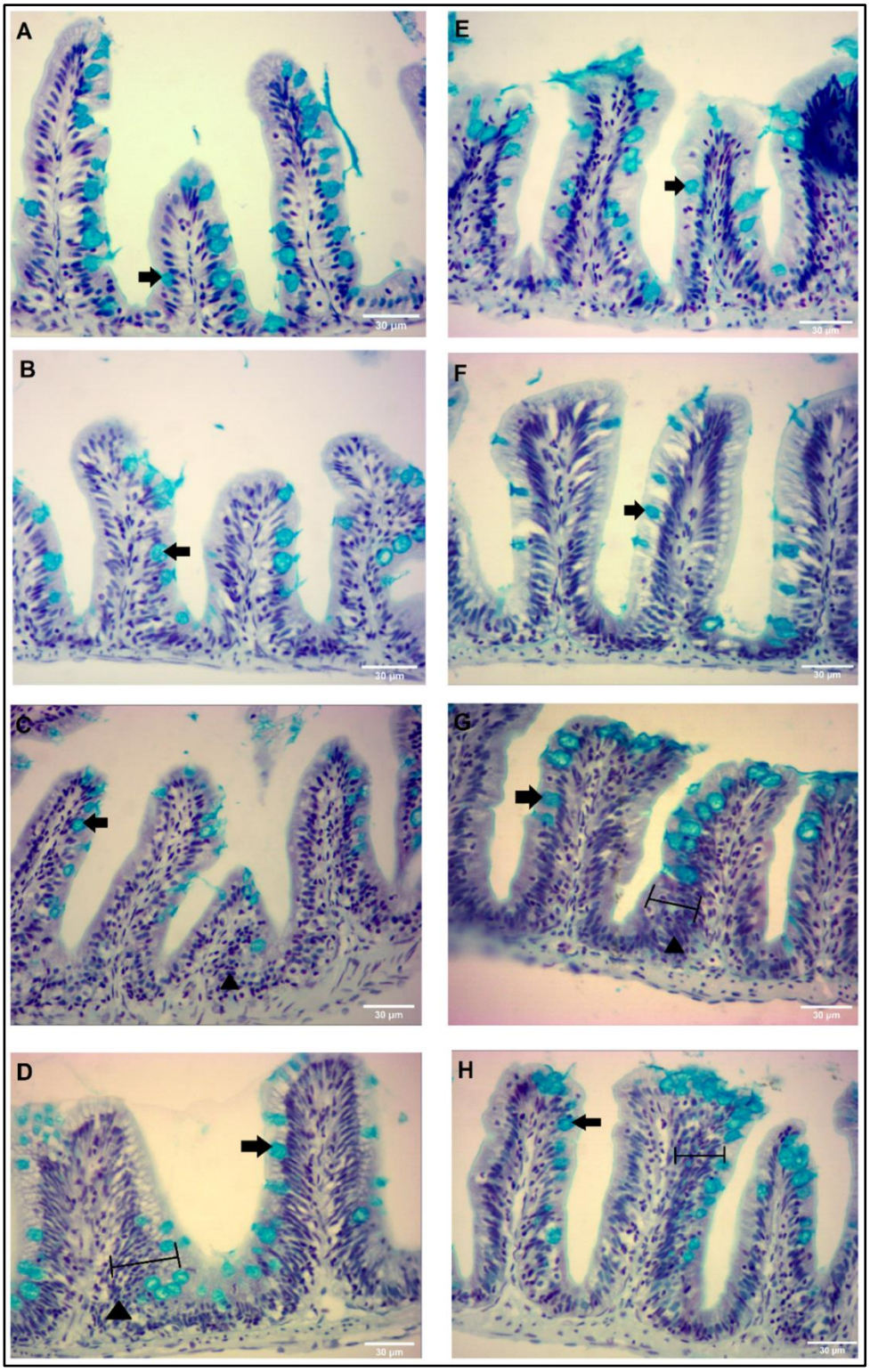
Source: Authors' private collection (2021)
Caption: Data are expressed as mean ± standard error. NTU: Nephelometric Turbidity Units; TiO_2-NPs: titanium dioxide nanoparticles.

3.3 Intestine histological analysis

It was observed a 100% survival rate of fish acutely and chronically exposed to 5.0 mg L^{-1}, 20 mg L^{-1} and 40 mg L^{-1} of TiO_2-NPs.

The histopathological alterations observed in the intestines (Figure 3) from the acute and chronic experiments were increase in the thickness of intestinal villi and leukocyte infiltrate, besides the goblet cells quantification.

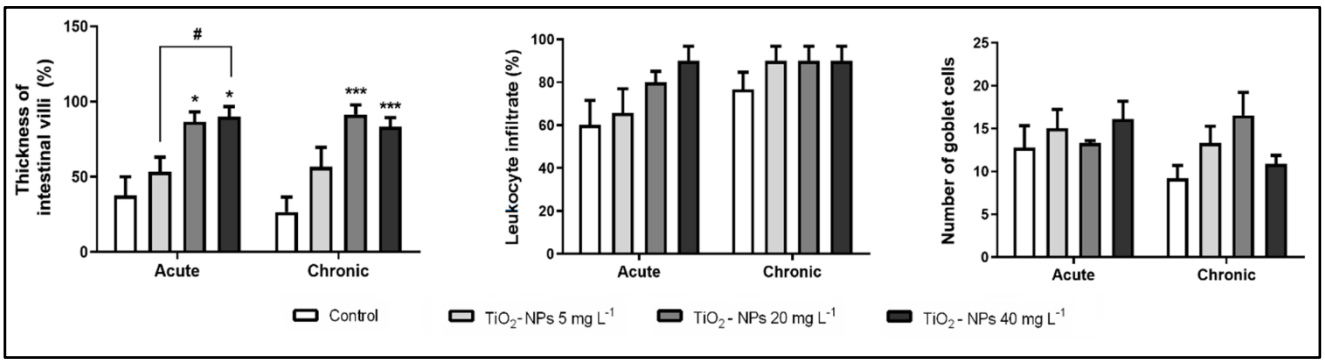
Figure 3 – Intestinal villi of *Danio rerio* exposed to different concentrations of TiO_2-NPs



Source: Authors' private collection (2021)
Caption: A to D: Acute exposure. A) Control group; B) 5.0 mg L^{-1} TiO_2-NPs; C) 20 mg L^{-1} TiO_2-NPs; D) 40 mg L^{-1} TiO_2-NPs. E to H: Chronic exposure. E) Control group; F) 5.0 mg L^{-1} TiO_2-NPs; G) 20 mg L^{-1} TiO_2-NPs; H) 40 mg L^{-1} TiO_2-NPs. Black arrows indicate goblet cells stained with Alcian blue, arrow heads point leukocyte infiltrate, and the bars (|---|) indicate an increase of intestinal villi.

Regarding to the thickness of intestinal villi, it was observed a significant increase in animals from the higher concentrations 20 mg L^{-1} (p = 0.033) and 40 mg L^{-1} (p = 0.001) of TiO_2-NPs compared to the control group of the acute exposure (Figure 4). In the chronic exposure, the same concentrations, 20 mg L^{-1} (p = 0.0001) and 40 mg L^{-1} (p = 0.0006) of TiO_2-NPs, differed from the control group. However, there were no significant differences comparing the acute and chronic exposure of animals exposed to 5.0 mg L^{-1} (p = 0.9985), 20 mg L^{-1} (p = 0.9939) and 40 mg L^{-1} (p = 0.9784) of TiO_2-NPs. Regarding the leukocyte infiltrate in the intestinal epithelium, there were no statistical differences among the groups in the acute (p = 0.584) and chronic (p = 0.83) exposures. There were also no significant differences among concentrations, 5.0 mg L^{-1} (p = 0.16), 20 mg L^{-1} (p = 0.8649) and 40 mg L^{-1} (p = > 0.9999) of TiO_2-NPs comparing the acute and chronic exposure. For the goblet cells, there were no significant alterations neither in acute exposure (p = 0.619) nor in the chronic exposure (p = 0.644). In comparison with the acute exposure, there were no statistical differences in animals exposed to 5.0 mg L^{-1} (p = 0.955), 20 mg L^{-1} (p = 0.6852) and 40 mg L^{-1} (p = 0.2282) of TiO_2-NPs.

Figure 4 – Percentage of increased thickness of intestinal villi, leukocyte infiltrate and the number of goblet cells in *Danio rerio* acutely and chronically exposed to TiO_2-NPs



Source: Authors' private collection (2021)

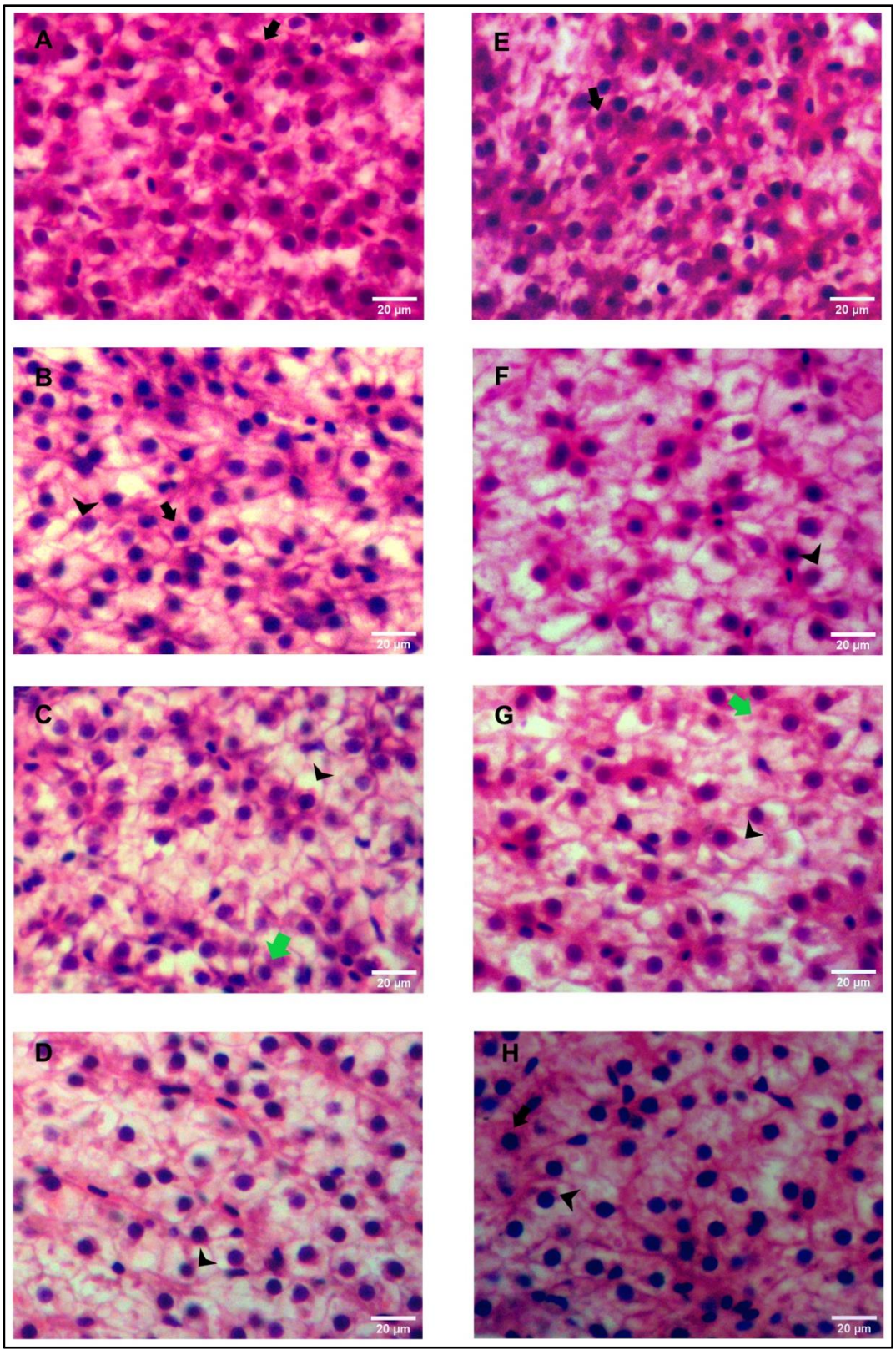
Caption: Data are expressed as mean ± standard error. Asterisks represent statistical differences compared to the control group (* = p < 0.05; ** = p < 0.01; *** = p < 0.001) and the pound indicates significant difference between treatments (p > 0.05; one-way ANOVA test followed by Tukey post-hoc for different treatments; Student's t-test for different exposure periods).

3.4 Liver histological analysis

The predominant histological findings observed in the liver (Figure 5) in the acute and chronic exposures were normal hepatocyte reduction, vacuolization, and peripheral nucleus.

There was a significant reduction in the normal hepatocytes percentage in animals exposed to 40 mg L^{-1} of TiO_2-NPs in the acute exposure compared to the control group ($p = 0.016$), as well as in the group chronically exposed to 5.0 mg L^{-1} of TiO_2-NPs ($p = 0.025$) (Figure 6). However, there were no differences comparing the acute and chronic exposure of the groups exposed to 5.0 mg L^{-1} ($p = 0.985$), 20 mg L^{-1} ($p = 0.899$), and 40 mg L^{-1} ($p = 0.887$) of TiO_2-NPs. About the hepatocyte vacuolization percentage, there was an increase in the group acutely exposed to 40 mg L^{-1} of TiO_2-NPs in relation to the control group ($p = 0.013$). Regarding the chronic exposure, statistical difference was only observed in animals exposed to 5.0 mg L^{-1} of TiO_2-NPs compared to the control group ($p = 0.032$). No significant differences were observed comparing the acute and chronic exposure in all concentrations: 5.0 mg L^{-1} ($p = 0.959$), 20 mg L^{-1} ($p = 0.876$) and 40 mg L^{-1} ($p = 0.235$) of TiO_2-NPs. For the peripheral nucleus percentage, a significant increase was observed in animals after acute exposure to 20 mg L^{-1} of TiO_2-NPs compared to the control group ($p < 0.0001$), whereas no differences among groups were observed after chronic exposure ($p = 0.11$). There was a significant increase in the peripheral nucleus percentage comparing acute and chronic exposure of animals acutely exposed to 20 mg L^{-1} of TiO_2-NPs ($p < 0.0001$), but no differences were observed in the groups exposed to 5.0 mg L^{-1} ($p = 0.311$) or 40 mg L^{-1} ($p = 0.999$).

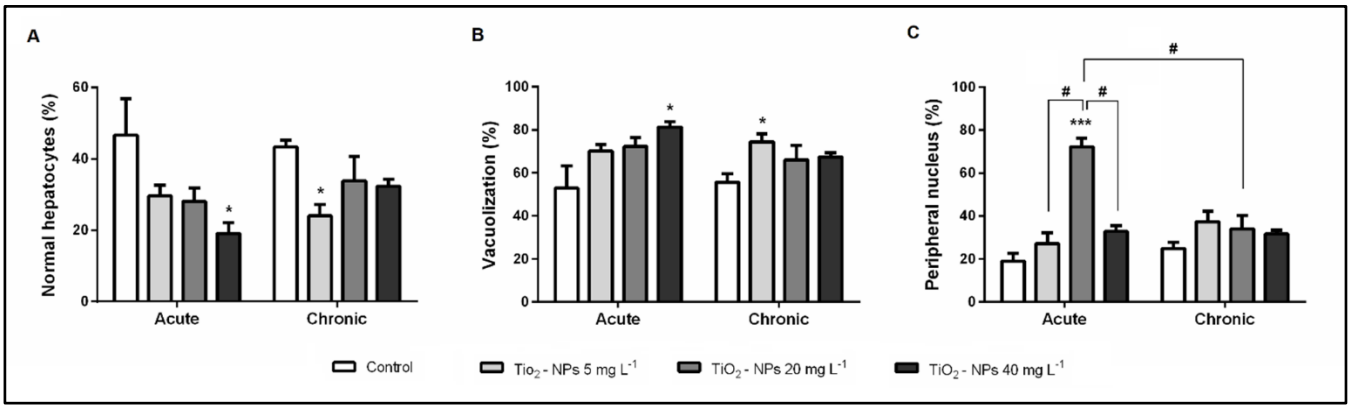
Figure 5 – Hepatocytes of *Danio rerio* exposed to different concentrations of TiO_2-NPs



Source: Authors' private collection (2021)

Caption: A to D: Acute exposure. A) Control group; B) 5.0 mg L^{-1} TiO_2-NPs; C) 20 mg L^{-1} TiO_2-NPs; D) 40 mg L^{-1} TiO_2-NPs. E to H: Chronic exposure. E) Control group; F) 5.0 mg L^{-1} TiO_2-NPs; G) 20 mg L^{-1} TiO_2-NPs; H) 40 mg L^{-1} TiO_2-NPs. Black arrows indicate normal hepatocytes, arrow head means vacuolization, and green arrows indicate cells with peripheral nucleus.

Figure 6 – Percentage of normal hepatocytes, vacuolization, and cells with peripheral nucleus in *Danio rerio* acutely and chronically exposed to TiO_2-NPs



Source: Authors' private collection (2021)
Caption: Data are expressed as mean ± standard error. Asterisks represent statistical differences compared to the control group (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) and the pound indicates significant difference between treatments or exposure periods ($p > 0.05$; one-way ANOVA test followed by Tukey post-hoc for different treatments; Student's t-test for different exposure periods).

3.5 Genotoxicity

Regarding the micronucleus test (Table 3), there was no significant difference among groups ($p = 0.967$) in the acute exposure. However, the animals exposed to 20 mg L^{-1} of TiO_2-NPs differed significantly from the control group ($p = 0.046$) in the chronic exposure. In the other groups, no significant differences were observed for the micronucleus counting ($p > 0.05$). There were also no significant differences between both exposures (acute and chronic) for the groups exposed to 5.0 mg L^{-1} ($p = 0.242$), 20 mg L^{-1} ($p = 0.145$) and 40 mg L^{-1} ($p = 0.242$) of TiO_2-NPs.

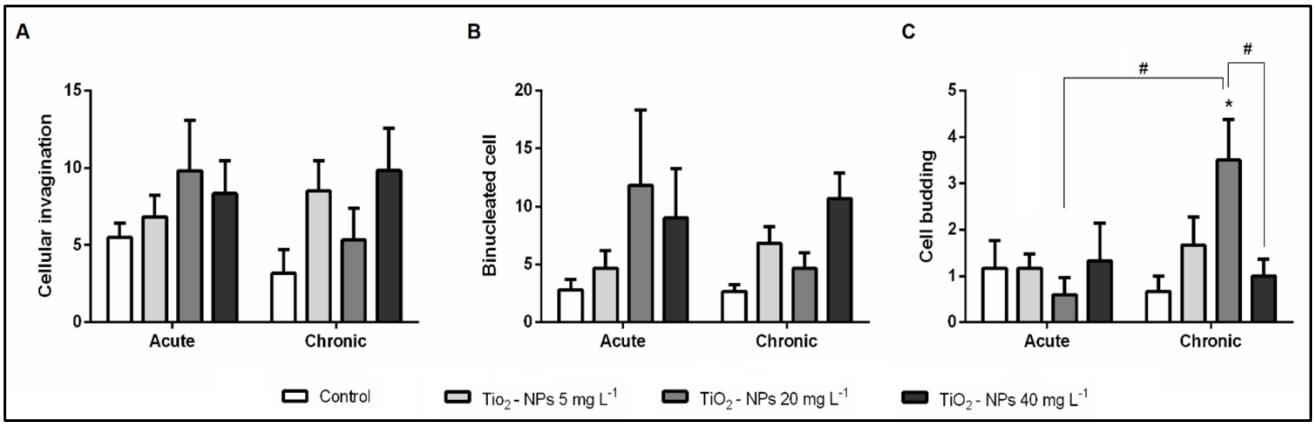
Table 3 – Micronucleus in erythrocytes of *Danio rerio* after acute and chronic exposure to TiO_2-NPs

	Control group	**5.0 mg L^{-1}**	**20 mg L^{-1}**	**40 mg L^{-1}**	**p**
Acute exposure	0.33 ± 0.51 [a]	0.50 ± 0.83 [a]	0.60 ± 1.34 [a]	0.50 ± 1.22 [a]	0.967
Chronic exposure	0.00 [a]	0.83 ± 0.40 [a]	1.00 ± 0.63 [b]	1.00 ± 0.89 [a]	0.046
p (Mann-Whitney)	-	0.242	0.145	0.242	-

Source: Authors' private collection (2021)
Caption: Data are expressed as mean ± standard deviation and different letters represent statistical difference among treatments ($p < 0.05$, Kuskal-Wallis test followed by Dunn's test for different treatments; Mann-Whitney test for different exposure periods).

In relation to nuclear abnormalities (Figure 7), no significant differences were observed among groups in the acute experiment for all evaluated abnormalities: cellular invagination ($p = 0.75$), binucleated cell ($p = 0.39$), and cell budding ($p = 0.28$). In the chronic exposure, there were also no statistical differences among groups for invagination ($p = 0.673$) and binucleation ($p = 0.704$), but it was observed a significant increase of cell budding in erythrocytes of zebrafish exposed to 20 mg L^{-1} of TiO_2-NPs compared to the control group ($p = 0.0072$). In contrast, animals exposed to 40 mg L^{-1} of TiO_2-NPs showed a significant reduction in the number of buddings compared to the group exposed to 20 mg L^{-1} of TiO_2-NPs ($p = 0.022$). There were no statistical differences for invagination comparing the acute and chronic exposure of the groups exposed to 5.0 mg L^{-1} ($p = 0.969$), 20 mg L^{-1} ($p = 0.464$) and 40 mg L^{-1} ($p = 0.979$) of TiO_2-NPs. There were also no significant differences for binucleation in 5.0 mg L^{-1} ($p = 0.978$), 20 mg L^{-1} ($p = 0.352$) and 40 mg L^{-1} ($p = 0.991$) groups comparing acute and chronic exposure. Nevertheless, a significant increase of cell budding was observed in the group chronically exposed to 20 mg L^{-1} of TiO_2-NPs in relation to the acute exposure ($p = 0.003$) of this same concentration. The groups exposed to 5.0 mg L^{-1} ($p = 0.955$) and 40 mg L^{-1} ($p = 0.99$) of TiO_2-NPs did not show significant difference for this abnormality.

Figure 7 - Nuclear abnormalities in erythrocytes of *Danio rerio* after acute and chronic exposure to TiO_2-NPs



Source: Authors' private collection (2021)
Caption: A) Celullar invagination; (B) Binucleated cell; (C) Cell budding. Data are expressed as mean ± standard error. Asterisks represent statistical differences compared to the control group and the pound means statistical differences between treatments or exposure periods ($p < 0.05$, one-way ANOVA test followed by Tukey post-hoc for different treatments; Mann-Whitney test for different exposure periods).

4 DISCUSSION

Titanium dioxide nanoparticles are being rapidly introduced into the food industry in the anatase and/or rutile phases (YANG *et al*., 2014). The anatase crystalline form, considered the most used in food, is more reactive and has greater photocatalytic activity, which generally makes it more cytotoxic than rutile (ROMPELBERG *et al*., 2016; SAYES *et al*., 2006). In some cases, the anatase/rutile mixture, that is used as food additive, may exhibit even greater toxicity than the individual phases (GERLOFF *et al*., 2012; GURR *et al*., 2005), by transferring photoinduced electrons from anatase to rutile (KAWAHARA *et al*., 2003). These additive effects are possible when the percentage of anatase is higher than rutile (ISWARYA *et al*., 2015), suggesting that the histopathological and genotoxic damages observed in our study may be associated with both polymers (anatase and rutile) of the mixture.

A common behavior of TiO_2-NPs is the tendency to aggregate and agglomerate, which was previously observed through SEM and transmission

electron microscopy (TEM) in several studies (CARMO *et al*., 2019; LEE *et al*., 2019; VIGNARDI *et al*., 2015; WANG; LUO; YAN, 2018). In the DLS analysis, the particle sizes of TiO_2-NPs corresponded to the estimated size by SEM due to N,N-dimethylformamide, which has previously demonstrated capable of turning the samples stable and monodisperse (TERISSE *et al*., 2013). However, DLS measurements usually present larger sizes in water solutions as dispersed nanoparticles can rapidly agglomerate because of physicochemical properties of the water and the surface charge of TiO_2-NPs (ATES *et al*., 2013; STROBEL *et al*., 2014).

Regarding water turbidity, some studies have already reported visible turbidity in TiO_2-NPs suspensions caused by large nanoparticle agglomerates that can settle out by gravity (EPA, 2010). Several factors may have contributed to the agglomeration, such as pH, ionic strength, temperature, water hardness, presence of microorganisms and natural organic matter (GRILLO; ROSA; FRACETO, 2015), but it is worth mentioning that the maintenance of most of these parameters is essential for the quality of life of *Danio rerio*. In these cases, TiO_2-NPs concentration in the liquid phase is affected and might be different from the concentration calculated from added TiO_2-NPs (VELZEBOER *et al*., 2008). Baalousha (2009) observed, in his study with iron oxide nanoparticles, that the sedimentation rate of the particles seems to be directly proportional to the initial concentration. Considering that the gills have direct contact with the bloodstream (WILSON; LAURENT, 2002), this concentration difference could have mainly affected genotoxic effects in 40 mg L^{-1} of TiO_2-NPs, which is very superior to our other concentrations.

The exposure to toxic agents does not always result in the death of aquatic organisms, especially when they are exposed to non-lethal concentrations (ARENZON *et al*., 2013). In this sense, our data agree with the literature, confirming the low lethality of TiO_2-NPs in acute exposures. Clemente *et al*. (2014) obtained LC_{50} values (96 hours) > 100 mg L^{-1} of TiO_2-NPs mixture in *Danio rerio* embryos,

which was also observed in *Carassius auratus* (ATES *et al*., 2013). For long-term experiments, the information available in the literature is still scarce, especially for fish exposed to TiO_2-NPs suspensions. In *Prochilodus lineatus*, subchronic exposure (14 days) to 1, 5, 10 and 50 mg L^{-1} of TiO_2-NPs did not result in the death of any animal (CARMO *et al*., 2019), while another study with adult *Danio rerio* reported significant mortality from fish chronically exposed (6 months) to 7 mg L^{-1} of TiO_2-NPs (CHEN *et al*., 2011). Thus, even if chronic exposure (30 days) to 5, 20 and 40 mg L^{-1} of TiO_2-NPs did not cause the death of *Danio rerio*, these concentrations may be lethal in longer exposures.

In toxicological studies, the intestine is a relevant organ because of being the first physiological barrier for orally ingested chemicals that can be potentially toxic (RYU *et al*., 2018). In the present study, there was an increase in the thickness of intestinal villi, also called as hyperplasia, in 20 and 40 mg L^{-1} of TiO_2-NPs in the acute and chronic exposures. This hyperplasia may be due to an enterocyte adaptation after suffering an injury stimulated by the higher concentrations of TiO_2-NPs, which can reduce the absorption of nutrients (AMMENDOLIA *et al*., 2017). Guo *et al*. (2017) observed that TiO_2-NPs caused an impact on the transport of iron, zinc, and fatty acids in Caco-2 cells, but no signs of inflammation or major alterations were observed. In the present study, there was no significant difference in the amount of goblet cells, so as Blevins *et al*. (2019) in their study with rats orally exposed to E171 for 100 days. In the small intestine of *Danio rerio*, goblet cells play an important role in the immune responses against foreign antigens, indicating that the amount of goblet cells was not sufficient to avoid intestinal damage, but was efficient in protecting the epithelium from inflammation during both exposure times (MENKE *et al*., 2011).

Regarding the intestinal absorption of particles, it was believed that particles larger than 50 nm would have an insignificant diffusion rate in the gastrointestinal tract of mammals because of the goblet cells, but actually pores within the mucus layer can pass particles up to 500 nm (JOVANOVIĆ, 2015; LAI *et al*., 2007). As

reported by Chen *et al*. (2013), TiO_2-NPs are able to cross the gastrointestinal tract and distribute to other organs, leading to a gradual accumulation in the whole body. In *Carassius auratus*, the exposure to 10 and 100 mg L^{-1} of TiO_2-NPs for 5 days promoted Ti bioaccumulation in the intestine and gills (ATES *et al*., 2013). In another study, *Danio rerio* orally exposed to 200 and 400 µg of TiO_2-NPs for 7 and 14 days presented Ti bioaccumulation in the intestine and liver, along with apical vacuolization and distal rupture of the epithelial cells of the intestinal villi, although there were no changes in other layers of the intestinal wall (CUNHA; BRITO-GITIRANA, 2020). These results indicate that even short exposure times can promote some histopathological changes in the intestine due to Ti bioaccumulation, while major alterations can be restrained by the goblet cell barrier.

Some other *in vivo* studies have evaluated the effect of TiO_2-NPs in the intestine after long-term exposures (MU *et al*., 2019; ZHU *et al*., 2020). Urrutia-Ortega *et al*. (2016) observed an increase in tumor formation in a colitis associated cancer (CAC) model after intragastric exposure to E171 for 11 weeks, along with over-expression of p65-NF-kB and reduced number of goblet cells in the colon tissue. In accordance, Bettini *et al*. (2017) noted an increase in the number of aberrant crypts as well as the number of large colonic aberrant crypt foci (ACF) in the colon of a chemically induced carcinogenesis model treated with E171 (10 mg kg^{-1}) for 100 days. These animals also presented an increase in TNF-α, IL-8 and IL-10 levels, which was not observed in rats treated with E171 for 7 days at the same dosage (BETTINI *et al*., 2017). These findings suggest that longer exposures to TiO_2-NPs could have promoted goblet cell reduction and consequently inflammation and carcinogenesis, although these parameters have not been evaluated in fish experiments for such exposure times yet.

The liver is another highly investigated organ because of its biotransformation reactions that can increase toxicity and carcinogenicity of metabolites (WOLF; WHEELER, 2018). One of the main alterations observed in the hepatic tissue is vacuolization, differentiated in glycogen-type, which has a

flocculent, angular, and central nucleus, and lipid-type, which is characterized by a nucleus displacement to periphery (WOLF; WHEELER, 2018; WOLF; WOLFE, 2005). As in other studies (FEDERICI; SHAW; HANDY, 2007; HAO; WANG; XING, 2009; MORGAN *et al*., 2018; ORAZIZADEH *et al*., 2014), an increase of both types of vacuolization was observed in hepatocytes of *Danio rerio* after TiO_2-NPs exposure, as well as the decrease of normal hepatocytes because of tissue alteration. The results presented in the acute exposure mean that the highest concentration is already capable of causing changes in a 96-hour exposure. In the chronic exposure, histological alterations were only observed in the lowest concentration, and not in 20 and 40 mg L^{-1} of TiO_2-NPs. This is possibly because of an intense particle agglomeration in the highest concentrations, which usually turn them less mobile and make them interact with suspended and sedimentary organic matter (CRANE *et al*., 2008; POWELL *et al*., 2010).

Previous studies have shown that TiO_2-NPs can accumulate in different organs, mainly the liver (HERINGA *et al*., 2018). Chen *et al*. (2011) investigated the chronic toxicity (2, 4 and 6 months) of TiO_2-NPs (1, 2, 4, 5 and 7 mg L^{-1}) in *Danio rerio* and observed high amounts of Ti in the liver and gills, besides an increase in liver weight in a concentration and time dependent manner that results in liver damage and can lead to lipidosis. A similar result was found by Federici, Shaw, and Handy (2007), who evaluated liver histology of *Oncorhynchus mykiss* exposed to 0.1, 0.5 and 1 mg L^{-1} of TiO_2-NPs and observed histological changes, such as: loss of sinusoid space, necrotic cells, cells with condensed nuclear bodies (apoptotic bodies) and cells showing nuclear division with condensed nuclear material. The authors also observed a few foci of lipidosis with minor fatty change similar to the vacuolization found in our study (FEDERICI; SHAW; HANDY, 2007). These studies demonstrate that Ti is capable of bioaccumulating in the liver even in lower concentrations of TiO_2-NPs, and it reflects the histopathological changes presented in the liver.

Although the mechanisms of TiO_2-NPs hepatic toxicity are not yet clear, some biochemical alterations are being associated with liver alterations (JIA *et al*., 2017; TUNÇSOY, 2021). In a study carried out by Hajirezaee, Mohammadi, and Naserabad (2020), *Cyprinus carpio* exposed to 0.125 mg L^{-1} of TiO_2-NPs for 21 days presented histological changes in the liver, such as: cytoplasm vacuolization, hepatocyte cloudy swelling, hypertrophy of the hepatocytes and nucleus atrophy. The animals also showed a reduction in SOD activity, and higher levels of cortisol, glucose, triglycerides, and liver damage biomarkers (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) (HAJIREZAEE; MOHAMMADI; NASERABAD, 2020). These results clarify that TiO_2-NPs affect energy and lipid metabolism, which could elucidate the increase in cell vacuolization observed in our study.

TiO_2-NPs demonstrate, in different studies, their ability to induce genotoxicity as a result of the exposure (JUGAN *et al*., 2012; LI *et al*., 2017; FILHO *et al*., 2019). However, conflicting results have been reported in micronucleus test. Positive and negative results were observed, for example, in human bronchial epithelial cells (BEAS-2B) (GURR *et al*., 2005; PRASAD *et al*., 2013) and human lymphocytes (TAVARES *et al*., 2014). The data setback can be explained by the use of different nanoparticles and means of preparation of the suspensions, causing different biological activities (MURUGADOSS *et al*., 2020). Another bias for different genotoxicity results in proliferative cells is the exposure time, considering that nanoparticles seem to require longer time for cell penetration and internalization than other chemical agents (KARLSSON *et al*., 2015). It means that the acute exposure time (96 hours) was not enough to cause chromosomal damage in *Danio rerio* erythrocytes. At the same time, these results reinforce the need to also assess chronic exposures to understand the real extent of the damage.

The micronucleus test is an important *in vitro* and *in vivo* biomarker of numerical and structural chromosomal changes caused by major damage to genetic material (HAYASHI, 2016). During cell division, DNA double-strand breaks can permit the separation of whole or fragmented chromosomes from chromatin,

giving rise to micronuclei (FENECH *et al*., 2003). This alteration may be associated with unregulated proliferation and anchorage-independent cell growth, which can lead to mutagenesis and carcinogenesis (HUANG *et al*., 2009). Our results are in accordance with studies *in vitro* (DEMIR *et al*., 2015) and *in vivo* (TROUILLER *et al*., 2009), which reported the occurrence of micronuclei after TiO_2-NPs exposure.

Through nuclear abnormalities, it is possible to identify clastogenic agents, which cause chromosomal breakage, and aneugenic agents, which induce aneuploidy during cell division (STICE *et al*., 2019). Some studies have shown that TiO_2-NPs exposure can induce clastogenic and aneugenic effects by the occurrence of binucleated cells and cell budding, for example (FILHO *et al*., 2019; VIDYA; CHITRA, 2018; VIGNARDI *et al*., 2015). However, the induction process of these structures is not yet fully understood.

Cell budding is characterized by a small evagination of the nuclear envelope containing euchromatin (CARRASCO; TILBURY; MYERS, 1990). Its formation represents DNA amplification and chromosomal instability (FENECH *et al*., 2011), which may be the result of a possible interaction between TiO_2-NPs and components of the mitotic spindle or proteins involved in chromosomal segregation (BIOLA-CLIER *et al*., 2020; HUANG *et al*., 2009). Budding can also become micronuclei (FENECH *et al*., 2011), which may be the reason for the increase of this abnormality in our study. In addition, the increase in buds between exposure times can be justified by a cell cycle delay caused by TiO_2-NPs after cell damage, accumulating a greater number of cells with buds over time (MEDINA-REYES *et al*., 2015).

Besides the interaction with DNA or non-DNA components, TiO_2-NPs genotoxicity can be also mediated by reactive oxygen species (ROS) through nanoparticle catalytic activity or chronic inflammation (MAGDOLENOVA *et al*., 2012; WANI; SHADAB, 2020). In zebrafish, the increase in ROS after chronic exposure to TiO_2-NPs is observed along with changes in antioxidant enzymes, such as glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD)

(BAR-ILAN *et al*., 2012; TANG; ZHANG; ZHU, 2019). The redox imbalance can result in oxidation of cellular components, which affects cell integrity and alters mechanisms such as signaling, proliferation and cell death (PURUSHOTHAMAN *et al*., 2014; WANI; SHADAB, 2020). In nanoparticle-induced inflammation, neutrophils and macrophages produce ROS via an inflammation-signaling pathway, resulting in lipid peroxidation and consequently tissue damage (BRAND *et al*., 2020).

Results presented in this study indicate that TiO_2-NPs in concentrations for human use are harmful to different organs of zebrafish in different exposure times and cause genotoxic damage, especially after chronic exposures. This represents human exposure time, as TiO_2-NPs are present in food and products of daily use, and are constantly reaching the environment. In this sense, more studies are necessary to assess histopathological and genotoxic effects of TiO_2-NPs and possible mechanisms involved in acute and chronic toxicity of this nanoparticle. Despite the wide range of authors reporting pathological changes in organisms exposed to TiO_2-NPs (AMMENDOLIA *et al*., 2017; BETTINI *et al*., 2017; CHEN *et al*., 2011; HERINGA *et al*., 2018; KOTIL; AKBULUT; YÖN, 2017; LI *et al*., 2019; TANG; ZHANG; ZHU, 2019; URRUTIA-ORTEGA *et al*., 2016), the European Food Safety Authority (EFSA, 2016) concluded that the consumer exposure to E171 in their food uses are unlikely to cause health risks. However, the *Agence Nationale de Sécurité Sanitaire l'alimentation, de l'environnement et du travail* (ANSES, 2019) questioned this decision, reevaluated studies and decided to ban the use of TiO_2-NPs as a food additive in France due to its genotoxic potential, which is not yet true in other countries.

5 CONCLUSION

Titanium dioxide nanoparticles, in human consumption compatible concentrations, caused histopathological changes in the intestine and liver of *Danio rerio* after acute and chronic exposures, whereas genotoxicity was only

observed after chronic exposure to TiO_2-NPs in the micronucleus test. These data emphasize TiO_2-NPs toxicity on different tissues of zebrafish, so its widespread use and lack of regulation in food products cause concerns regarding the health of its consumers.

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