Environmental Science and Pollution Research (2022) 29:62208–62218
https://doi.org/10.1007/s11356-021-17645-6

TiO_2-NPs and cadmium co-exposure: *in vitro* assessment of genetic and genomic DNA damage on *Dicentrarchus labrax* embryonic cells

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Received: 29 June 2021 / Accepted: 16 November 2021 / Published online: 26 November 2021

Abstract

The increased titanium dioxide nanoparticles (TiO_2-NPs) spread and their interaction with organic and inorganic pollutants arouses concern for the potential hazards for organisms and environment. This study tested in vitro the genotoxic effects of TiO_2-NPs (1 µg/mL) and cadmium (Cd) (0.1 µg/mL) co-exposure using *Dicentrarchus labrax* embryonic cells (DLEC) as experimental model. The genotoxicity tests (Comet assay, Diffusion Assay and Random Amplification of Polymorphic DNA (RAPD-PCR) were conducted after 3, 24 and 48 hours of exposure to TiO_2-NPs and Cd alone and in combination. The results showed that the percentage of DNA damage and apoptotic cells increases following 48 hours TiO_2-NPs exposure, while DNA instability was detected for all the times tested. Cd induced genotoxic effects starting from 3 hour-exposure and for all the treatment times. Cd + TiO_2-NPs co-exposure did not cause any genomic damage or apoptosis for all the exposure times. The possibility that Cd and TiO_2-NPs form aggregates no longer able of penetrating the nucleus and damaging the genetic material is discussed.

Keywords Titanium dioxide nanoparticles · Cadmium · DNA damage · Contaminant absorption · *Dicentrarchus labrax* embryonic cells (DLEC) · Environmental pollution

Introduction

Globalization and industrial development have caused the release into the environment of substances potentially harmful to animals and humans (Landrigan et al. 2016). Over the years, nanotechnology guaranteed a productive advantage in various industrial sectors, such as in diagnostic, medicine and pharmacology (Bayda et al. 2019). Among nanoparticles (NPs), titanium dioxide (TiO_2)-NPs are the most versatile used worldwide in many applications, and for this reason the attention was focused on the potential harmful effects related to their exposure (Grande and Tucci 2016). TiO_2-NPs are in the top five NPs used in consumer products, such as white pigment in paints, ceramics, as a food additive, in food packaging material, sunscreens, cosmetic creams, and as a component of surgical implants (Weir et al. 2012; Kong et al. 2018). The extensive usage of TiO_2-NPs in various commercial and industrial products has increased the risk of its potential environmental release confirming the investigation of potential damages humans and other living organisms can incur (Warheit and Donner 2015).

Responsible Editor: Cinta Porte

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Several *in vitro* and *in vivo* studies have explored three possible mechanisms through which TiO_2-NPs may exert a toxic effect: oxidative stress, inflammation, and apoptosis. These pathogenic mechanisms could or not be related (Ze et al. 2014; Gui et al. 2013; Hong et al. 2016; Abbasi-Oshaghi et al. 2019).

TiO_2-NPs have been reported to be internalized into cells by the immune response mediated by macrophages and neutrophils activation, which in turn results in increased reactive oxygen species (ROS) production with genotoxic and carcinogenic effects (Masoud et al. 2015). ROS accumulation induced by individual exposures to genotoxic pollutants causes direct DNA damage through the formation of 8-oxo guanine and genomic instability, which underlies the onset of cancer (Srinivas et al. 2019), resulting dangerous for development of aquatic organisms (Mottola et al. 2020). An *in vitro* investigation on aquatic cell model, *Dicentrarchus labrax* embryonic cells (DLEC), has shown that TiO_2-NPs increased the expression of inflammation-related genes such as interleukin- 8 (IL-8), even transforming growth factor-β (TGF-β), indicating that the apoptotic response induced by TiO_2-NPs could be caspase-3 independent (Picchietti et al. 2017). Furthermore, considering that TiO_2-NPs have been never detected inside the nucleus, the genetic damage in DLEC likely occurred indirectly through the ROS production (Picchietti et al. 2017). Oxidative stress was also observed in other aquatic models after exposure to high TiO_2-NPs concentrations in *Cyprinus carpio* (Hao et al. 2009) and an increase in glutathione (GSH) production has been reported as a potential mechanism to control the ROS levels generated from TiO_2-NPs in neotropical freshwater fish (Carno et al. 2018). Furthermore, the formation of hydroxyl radicals was the main mechanism of TiO_2-NPs toxicity observed in E. *coli* (Pathakoti et al. 2019).

In addition to NPs toxicity, potential interactions with other contaminants are of crucial importance in assessing the environmental risks of NPs (Sharma et al. 2019; Hartmann et al. 2010). In fact, peculiar feature of NPs is their surface / mass ratio, which is much larger than that of other particles, gives them the ability to adsorb and transport other compounds (De Jong and Borm 2008). Furthermore, when natural organic matter (NOM), ions and polysaccharides, meet the NPs, they adsorb on their surface and form a corona that alters the properties of the NP surface affecting their absorption (Crandon et al. 2020).

Among contaminants, heavy metals besides being naturally present in the environment, are found at higher concentrations due to anthropic activity, and even at low concentrations they represent a serious threat to living organisms including humans (Tchounwou et al. 2012); however, the consequences of their interaction with TiO_2-NPs are the subject of controversy.

The evidence of the inability to accurately establish the effects of the TiO_2-NPs interaction with other pollutants, allowed concluding that the toxic effects due TiO_2-NPs and chemicals interaction can be classified into 4 main categories: 1) increased accumulation and toxicity, 2) decreased accumulation and increased toxicity, 3) decreased accumulation and toxicity, 4) no change in accumulation and toxicity (Abdel-Latif et al. 2020).

The toxic effects of environmental pollution due to heavy metals, including cadmium (Cd), also appear to be mainly due to the indirect production of ROS (Wu et al. 2016) which in turn may cause human diseases such as infertility (Roychoudhury et al. 2021). Cadmium is known to cause indirect oxidative damage on DNA, through the production of ROS and to block DNA repair mechanisms thus causing genomic instability in humans (Rani et al. 2014). In fact, cadmium acts on the mitochondria generating ROS and triggering various related mechanisms such as the activation of apoptosis, alteration of gene expression, and mitochondrial permeability, with the consequent development of pathologies in humans (Genchi et al. 2020).

TiO_2-NPs can reach the aquatic environment through industrial discharges and cause bioaccumulation and biomagnification through the food chain of other pollutants already existent including heavy metals (Asztemborska et al.2018).

In the aquatic environment, TiO_2-NPs were found to interact with heavy metals causing the formation of a hazardous mix accumulated by aquatic organisms (Abdel-Latif et al. 2020). This effect called "*Trojan horse*" defines the potential capacity of nanomaterials (NMs) to transport chemicals and facilitate their absorption modifying their toxicity (Naasz et al. 2018). However, as for many other NMs, controversial results have been reported on the TiO_2-NPs-Cd *Trojan horse* effect: TiO_2-NPs significantly enhanced Cd accumulation, toxicity and DNA damage in the freshwater gastropod *Bellamya aeruginosa* (Ma et al. 2017), as well as in carp (Zhang et al. 2007). The combined toxicity of nanoparticles and heavy metals was observed in the presence of surfactants also in bacteria (Li et al. 2018), and such study suggested that the toxic effects of the combination of TiO_2-NPs and Cd depended on the Cd dose. In fact, the toxicity was reduced at lower Cd concentrations and increased at higher ones. On the contrary, the interaction between Cd and TiO_2-NPs was reported not to affect toxicity and genotoxicity in the Mediterranean mussel (Balbi et al. 2014), in sea bass (Nigro et al. 2015) and in the green algae *Chlamydomonas reinhardtii* (Yang et al. 2012), in which, toxicity and accumulation of Cd even decreased in presence of TiO_2-NPs.

For these reasons, the ability of TiO_2-NPs to influence the known toxicity of Cd raises concerns for the health of exposed organisms, especially in aquatic environments.

The present study is expected to elucidate the genotoxic effects due to TiO_2-NPs in combination with cadmium by investigating DNA damage induced by TiO_2-NPs (1 µg/mL) and Cd (0.1 µg/mL) alone and in combination for 3, 24 and 48 exposure hours (h) in an *in vitro* aquatic model (DLEC). DLEC cell line represents a good *in vitro* experimental model to assess the genotoxic effects by detecting DNA alterations in environmental genotoxic studies (Rocco et al. 2014). Fish cells have many functions that are similar to those of mammalian cells, but the advantages of DLEC respect to mammalian cells are that they are standardizable, easy to handle with relatively low variability, can be directly exposed to environmental samples of different osmolarity, more convenient, and less laborious to use (Buonocore et al. 2006). The genotoxic effects of TiO_2-NPs, Cd and interaction between TiO_2-NPs and Cd were analyzed in term of DNA strand breaks, degree of apoptosis, DNA-induced mutations and genome stability.

Materials and Methods

Chemicals

Titanium dioxide nanoparticles (Aeroxide; Evonik Degussa, Essen, Germany; Lot. 614061098) have a dimensional average of 21 nm and represent a mixture of 75% rutile and 25% anatase forms with 99.9% declared purity. The preparation of the TiO_2-NPs stock solution (10.0 mg/L) was performed according to Rocco and collaborators (Rocco et al. 2015). Briefly, TiO_2-NPs solution has been sonicated in medium (Millipore) for 3 h (40 kHz frequency, Dr. Hielscher UP 200S, Germany) and UV–Vis spectra were obtained in the range of 200–600 nm by a Shimadzu UV-1700 double beam spectrophotometer. TiO_2-NPs solution was characterized by analytical and morphological assay (Nigro et al. 2015). Cadmium chloride (CAS number 10108-64-2, 99.999% purity) was provided by Sigma-Aldrich (St. Louis, Missouri, USA).

Particles' primary characterization

The particle suspension, after sonication, was dropped onto a formvar-coated copper grid and observed with a Jeol JEM 100 SX (Tokyo Japan) TEM at 80 kV, which revealed TiO_2-NP particles having a spheroid irregular shape with a mean diameter of 25 ± 6 nm (Fig. 1). Approximately 200 particles were measured.

Dicentrarchus Labrax Embryonic Cell Line (DLEC)

The continuous embryonic cell line established from *Dicentrarchus labrax* (DLEC) (Kerafast, US, Lot. ETS00) is the first embryonic cell line from the European sea bass

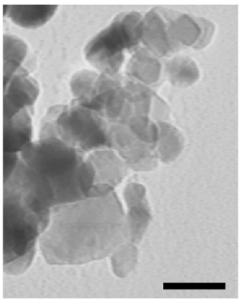

Fig. 1 TEM micrograph showing aggregation pattern of TiO_2-NPs (bar 50 nm).

(*Dicentrarchus labrax*). The DLEC were cultured ($5x10^3$ cells /cm^2) in plastic culture T75 flask (seal cap, surface of 25 cm^2) (BD Falcon) in Leibovitz L-15 medium (Sigma-Aldrich) added with 1% l-glutamine, 100 U/ml penicillin-streptomycin and 10% FBS at 22°C according to Buonocore and collaborators (Buonocore et al. 2006). When DLEC culture has reached cell confluence showing a fibroblast-like shape, the cells were trypsinized with 1 ml of trypsin-EDTA (200 mg/L) and split in five experimental groups. All buffers and solutions used for DLEC were brought to sea bass osmolarity (355 mOsm/kg) with 2M NaCl at 18 °C (Buonocore et al. 2006).

Experimental design

To investigate the *in vitro* genotoxic impact of TiO_2-NPs and Cd alone and in combination, the following experimental DLEC treatments were set up: one flask was treated with 1 µg/mL of TiO_2-NPs, one with 0.1 µg/mL of Cd, one with 1 µg/mL of TiO_2-NPs plus 0.1 µg/mL of Cd, untreated flask was used as negative control. Benzene 0.4 µL/mL, a well-known genotoxic agent, was used as positive control (Whysner et al. 2004). The growth kinetics of DLEC cells show a linear increase up to 96 h (Buonocore et al. 2006). In this study we selected minimum and intermediate times of DLEC cell growth (3, 24 and 48 h) to ensure a stable number of cells for the three exposure times. Cell incubation was explained above. Cd concentration was selected according to previous pilot study that highlighted Cd values causing significant biological responses under LC50 (Nigro et al. 2015). TiO_2-NPs concentration was selected according to our previous study (Rocco et al. 2015) in accordance with amount in aquatic environmental (Gottschalk et al. 2013). To obtain valid statistics three independent measurements were performed.

Cell viability

DLEC viability was assessed by Trypan blue staining (Strober 2001). Briefly, 0.4% dye was added to cell suspension on a slide. Through the analyses by optical microscope,

viable cells (clear cytoplasm) are distinguished by non-viable cells (blue cytoplasm).

Comet Assay

DLEC DNA damage was analyzed by Comet assay (Frenzilli et al. 2009). DLEC samples were trypsinized and centrifugation at 1000 rpm for 8 minutes, then the pellet was resuspended in 200 µl of PBS1X (355 mOsm/kg). DLEC suspension ($5*10^5$) (20 µl) have been mixed with 80 µl of Low Melting Point Agarose (LMPA) (0.75 %) and were placed in Normal Melting Agarose (1%) layers on slides at 37°C. After 30 minutes (min) at 4 °C, 100 µl of LMPA 0.5% was added on slides and incubated overnight in the cold lysis solution (NaCl 2.5 M, Na_2EDTA 0.1 M, Tris-Base 0.4 M, TRITON-X100 1%, DMSO 10%, pH 10). Then the slides were site for 10 min a mild alkaline solution (NaOH 10N, EDTA 200 mM, pH 12.1) and exposed to electrophoresis (25V,300 mA, 0.7 V/cm) for 15 min. Finally, the slides were washed with neutralizing solution (Tris-HCl 0,4 M, pH 7) and fixed in cold methanol. After staining with 30x ethidium bromide, the slides were observed by the fluorescence microscope (Nikon Eclipse E-600). The images were acquired with the "OpenComet" software (Gyori et al. 2014) and tail moment (tail moment = tail length x percentage (%) of DNA in the tail) was considered as genotoxicity parameter. For each experimental group, 100 cells were scored from three slides for a total of 300 cells. Three independent experiments were performed.

Diffusion Assay

The Diffusion Assay was performed as Comet Assay protocol with one difference: the electrophoretic run is not carried out. In non-electrophoretic conditions, apoptotic cells were identified by the presence of highly dispersed DNA giving rise to a characteristic halo around the nucleus (Singh 2000). In detail, apoptotic cell nuclei, characterized by the high dispersion of DNA (three times over the mean nucleus diameter) have a hazy or undefined outline without any clear boundary due to nucleosomal-sized DNA fusing into agarose. Necrotic cells nuclei are bigger and poorly defined. They have a clear, defined outer boundary of the DNA and a relatively homogenous appearance (Singh 2000). Cells without DNA damage (neither necrotic nor apoptotic cells) are clearly defined, while apoptotic nuclei have a larger size with a projection of DNA all around. Only class 5 cells (apoptotic cell) according to damage classes division reported by Cantafora and co-workers (Cantafora et al. 2014) were counted. At least 100 cells per data point were scored and the percentage of apoptotic cells evaluated. Diffusion assay data analysis was performed by three scorers blind to treatment. Three independent experiments were performed.

Random Amplification of Polymorphic DNA (RAPD-PCR) technique and Genome Template Stability (%) analysis

To assess genomic template stability percentage (GTS, %), Random Amplification of Polymorphic DNA (RAPD-PCR) according to Rocco and collaborators (Rocco et al. 2014) was performed. DLEC DNA was extracted from 200 µl of cell suspension by High pure PCR template preparation Kit (ROCHE Diagnostics) according to the producer's instructions. Briefly, 200 µl of sample cells, 200 µl Binding Buffer and 40 µl Proteinase K were mixed and incubated at 70°C for 10 min. Then, 100 µl Isopropanol were added, mixed and apply to a High Pure Filter Tube and centrifuge at 9000 rpm for 1 min. After the addition of inhibitor removal buffer and twice washes with Wash Buffer, 200 µl of pre-warmed Elution Buffer were added and centrifuged the tube for 1 min at 9000 rpm. The microcentrifuge tube contains the eluted purified DNA. Amplification was made in 25 µl reaction volumes containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 µM of primer 6 (5'-d[CCCGTCAGCA]-3') and 12.5 µl of Taq DNA Polymerase (Roche). The RAPD-PCR was conducted under the following conditions: one first step at 94°C for 2 minutes, then 1 min at 95 °C, 1 minutes at 36 °C and 2 minutes at 72°C, for 45 cycles. The RAPD-PCR products underwent electrophoresis (130 V) for 1 h on 2% agarose gel and were observed after gel staining with 1% ethidium bromide. A binary coded character (1, 0) was used for RAPD investigation and elaborated by Genesis software (Graz University of Technology Istitute for Genomics and Bioinformatics 1.8.1). Thanks to RAPD-PCR polymorphic patterns profiles, template genomic stability percentage (GTS %) was calculated as follows:

$$\mathrm{GTS} = (1 - a/n) \times 100,$$

where a is the average number of polymorphic bands found in each treated sample and n is the total number of bands in the negative control. The variations of polymorphic bands, considered as disappearance of bands and/or appearance of new bands respect to control profile, are assessed as a percentage respect to the negative control set to 100% (Rocco et al. 2015).

Transmission Electron Microscopy (TEM)

TEM analysis was performed as reported by Picchietti and co-workers (Picchietti et al. 2017). Briefly, DLEC cells were seeded on sterile PET track-etched membranes that were

inserted in 24- well cell culture plates (IWAKI, Scitech Div. Asahi Techno Glass). Cells were cultured overnight at 22 °C, in FBS-free L-15 medium and then exposed for 24 h to the treatments (see experimental design). Control was obtained adding fresh FBS-free medium for 24 h. After treatments, cells were fixed overnight at 4 °C with 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2. Samples were washed with cacodylate buffer, then post-fixed with 1% osmium tetroxide and 0.15% ruthenium red in 0.1 M cacodylate buffer at pH 7.2 for 1 h at 4 °C. After washing in distilled water, the samples were dehydrated in graded series of acetone and embedded in epon-based resin. Ultrathin sections (60-80 nm) were stained with 1% uranyl acetate and Reynolds lead citrate and then observed by TEM (JEOL 1200 EXII). Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped with the iTEM software.

Statistical analysis

The results were expressed as mean and standard deviation (SD). Differences in DLEC viability, DNA damage, degree of apoptosis and genomic stability among experimental groups were analyzed with ANalysis Of VAriance (ANOVA) though GraphPad Prism 6 software. Data were considered statistically significant for p-value (p) ≤ 0.05.

Results

Dicentrarchus Labrax Embryonic Cell Line (DLEC) viability

The exposure to TiO_2-NPs for 3, 24 and 48 h did not reduce DLEC viability, while Cd provoked a statistically significant viability reduction after 24 and 48 h. The treatment to TiO_2-NPs in combination with Cd did not induce a statistically significant variation in DLEC viability (Fig. 2).

Comet Assay

The data obtained by Comet Assay showed that TiO_2-NPs DLEC exposure caused a statistically significant DNA damage increase ($p \leq 0.05$) after 48 h; while Cd treatment induced a statistically significant increase ($p \leq 0.05$) of DNA strand breaks in DLEC for all the exposure times. As regards TiO_2-NPs and Cd co-exposure for 3, 24 and 48 h, the treatment did not determine any statistically significant increase of DNA damage compared to the negative control (Fig. 3).

Diffusion Assay

The results of Diffusion assay showed a statistically significant apoptotic cells induction ($p \leq 0.05$) exerted by TiO_2-NPs after 48 h exposure. The exposure to Cd after 3, 24 and 48 h induced a statistically significant increase ($p \leq 0.05$) of apoptotic DLEC (class 5) respect to controls. TiO_2-NPs plus Cd treatment did not induce any statistically significant

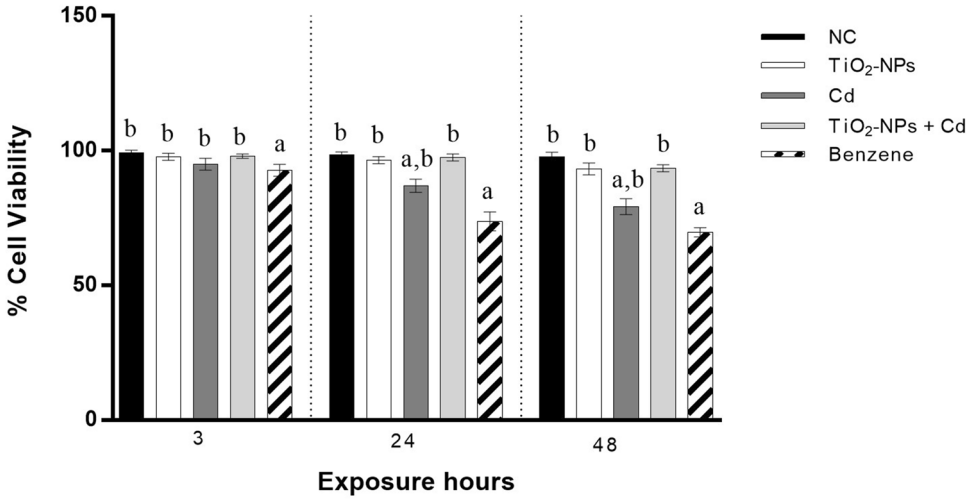

Fig. 2 Percentage of alive DLEC after 3, 24 and 48h exposure to TiO_2-NPs, Cd, TiO_2-NPs plus Cd and benzene. The dark bar is the negative control (NC); the white bar is TiO_2-NPs (1 µg/mL) treated cells; the dark grey bar is Cd (0.1 µg/mL) treated cells; the light grey bar is TiO_2-NPs (1 µg/mL) + Cd (0.1 µg/mL) co-treated cells; the striped bar is benzene (0.4 µL/mL, positive control) treated cells. Letters correspond to diverse statistical significances (ANOVA); a: $p \leq 0.05$ in comparison with NC; b: $p \leq 0.05$ in comparison with benzene exposure.

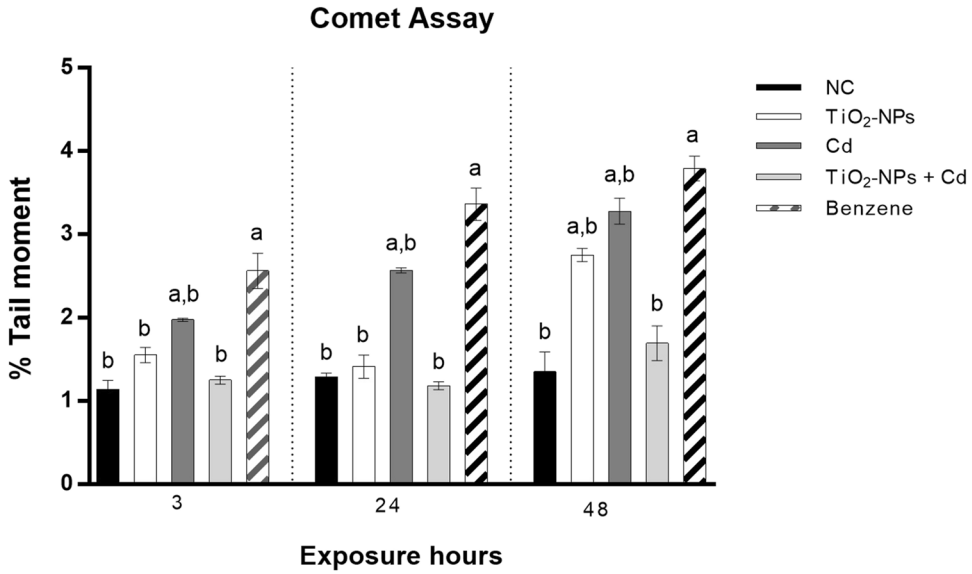

Fig. 3 Percentage tail moment in DLEC after 3, 24 and 48h exposure to TiO_2-NPs, Cd, TiO_2-NPs plus Cd and benzene. The dark bar is the negative control (NC); the white bar is TiO_2-NPs (1 µg/mL) treated cells; the dark grey bar is Cd (0.1 µg/mL) treated cells; the light grey bar is TiO_2-NPs (1 µg/mL) + Cd (0.1 µg/mL) co-treated cells; the striped bar is benzene (0.4 µL/mL, positive control) treated cells. Letters correspond to diverse statistical significances (ANOVA); a: $p \leq 0.05$ in comparison with NC; b: $p \leq 0.05$ in comparison with benzene exposure.

variation in percentage of apoptotic cells at all the tested exposure times (Fig. 4).

RAPD-PCR technique and Genome Template Stability (%) analysis

The RAPD-PCR analysis showed that 3 and 24 h exposure to TiO_2-NPs induced the loss of three bands and at 48 h induced the appearance and disappearance of two bands respect to the negative control, while 3, 24 and 48 h exposure to Cd provoked a variation of four bands compared to negative control. Polymorphic profiles of the DLEC DNA co-treated to TiO_2-NPs and Cd showed only one band change after 3-hour exposure respect to polymorphic profiles DNA of the untreated DLEC (Fig. 5).

Thanks to data from RAPD-PCR, the percentage of genome stability in DLEC exposed to TiO_2-NPs alone and in combination with Cd was assessed. TiO_2-NPs and Cd single exposure determined a statistically significant reduction ($p \leq 0.05$) in genomic stability after 3, 24 and 48 h,

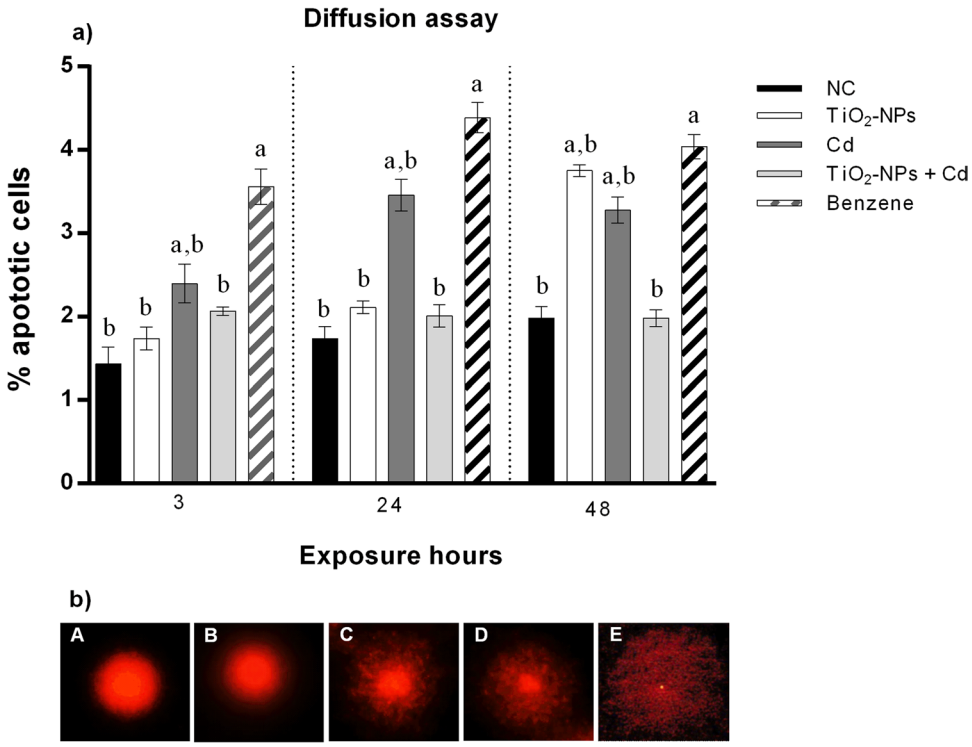

Fig. 4 (a) Percentage of apoptotic DLEC after 3, 24 and 48h exposure to TiO_2-NPs, Cd, TiO_2-NPs plus Cd and benzene. The dark bar is the negative control (NC); the white bar is TiO_2-NPs (1 µg/mL) treated cells; the dark grey bar is Cd (0.1 µg/mL) treated cells; the light grey bar is TiO_2-NPs (1 µg/mL) + Cd (0.1 µg/mL) co-treated cells; the striped bar is benzene (0.4 µL/mL, positive control) treated cells. Letters correspond to diverse statistical significances (ANOVA); a: $p \leq 0.05$ in comparison with NC; b: $p \leq 0.05$ in comparison with benzene exposure. (b) Photomicrographs of DLEC nuclei processed for the DNA Diffusion assay. Four normal cells at increasing degree of damaged DNA (A, B, C, D) and an apoptotic cell (E) are shown (magnification: 40×).

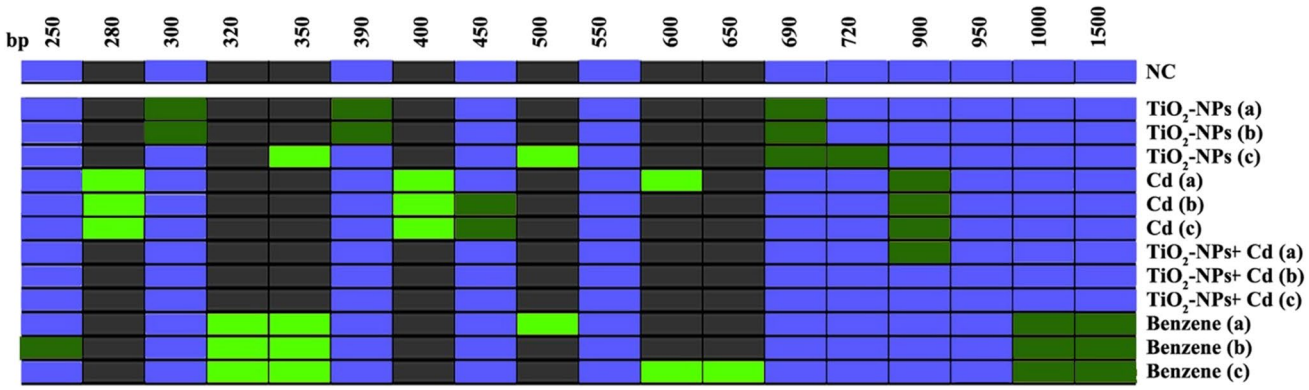

Fig. 5 Molecular sizes (bp) analyzed by Genesis software of appeared and disappeared bands after amplification with primer 6 in DLEC DNA exposed to 1 µg/mL TiO_2-NPs, 0.1 µg/mL Cd and 1 µg/mL TiO_2-NPs, 0.1 µg/mL Cd co-exposure, for a) 3 hours, b) 24 hours, c) 48 hours. In light green new bands appeared; in dark green disappeared bands; in blue not changed bands with respect to the control (NC).

whereas TiO_2-NPs and Cd co-exposure did not induce any statistically significant decrease in DLEC genomic stability (Fig. 6).

Cellular internalization of TiO_2-NPs

Ultrastructural analysis confirmed that TiO_2-NPs were readily *internalized* into the DLEC cells. Nps were found in the cytoplasmic vesicles and as agglomerates in the extracellular areas. When cells were co-exposed to TiO_2-Nps and Cd, Nps were taken up from the surrounding medium and trapped within cytoplasmic vesicles (Fig. 7A, B). TiO_2-Nps were not detected inside the nucleus. Control cells did not show any Nps (data not shown).

Discussion

Absorption of contaminants to TiO_2-NPs has been reported to increase the level of *in vivo* exposure in natural environments (Asztemborska et al. 2018) increasing the risk of oxidative damage, and for this reason most of the studies conducted on aquatic organisms focus on the increase in the accumulation of heavy metals and metalloids caused by TiO_2-NPs co-exposure. However, several studies show that TiO_2-NPs and Cd association leads to a damage reduction respect to the single substance exposure. The toxicity and genotoxicity modulation may be due to NPs ability to absorb and transport substances, which on one hand can increase damage through the *Trojan horse* mechanism, but on the other hand it reduces free pollutants concentration in the environment, leading to the formation of compounds, no

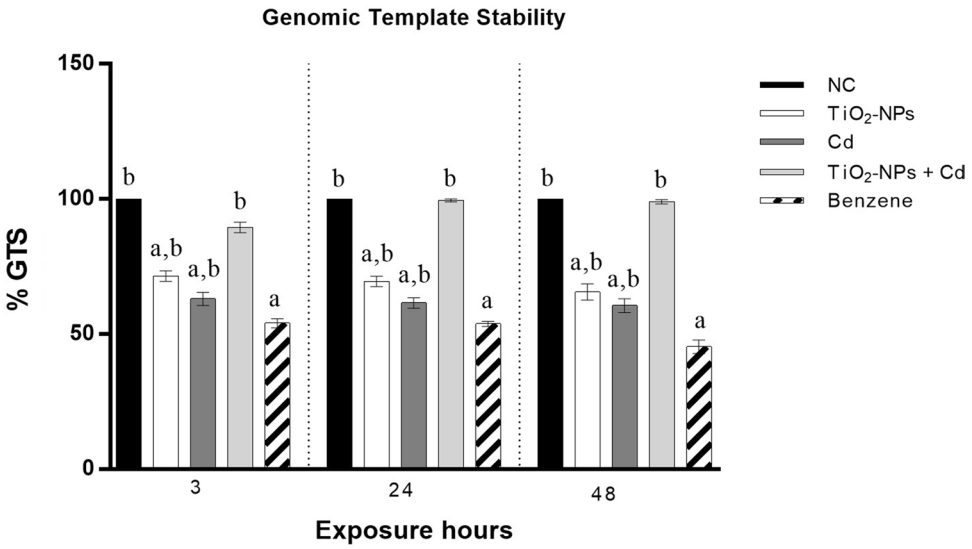

Fig. 6 Changes in percentage of Genome Template Stability in DLEC DNA after 3, 24 and 48h exposure to TiO_2-NPs, Cd, TiO_2-NPs plus Cd and benzene. The dark bar is the negative control (NC); the white bar is TiO_2-NPs (1 µg/mL) treated cells; the dark grey bar is Cd (0.1 µg/mL) treated cells; the light grey bar is TiO_2-NPs (1 µg/mL) + Cd (0.1 µg/mL) co-treated cells; the striped bar is benzene (0.4 µL/mL, positive control) treated cells. Letters correspond to diverse statistical significances (ANOVA); a: $p \leq 0.05$ in comparison with NC; b: $p \leq 0.05$ in comparison with benzene exposure.

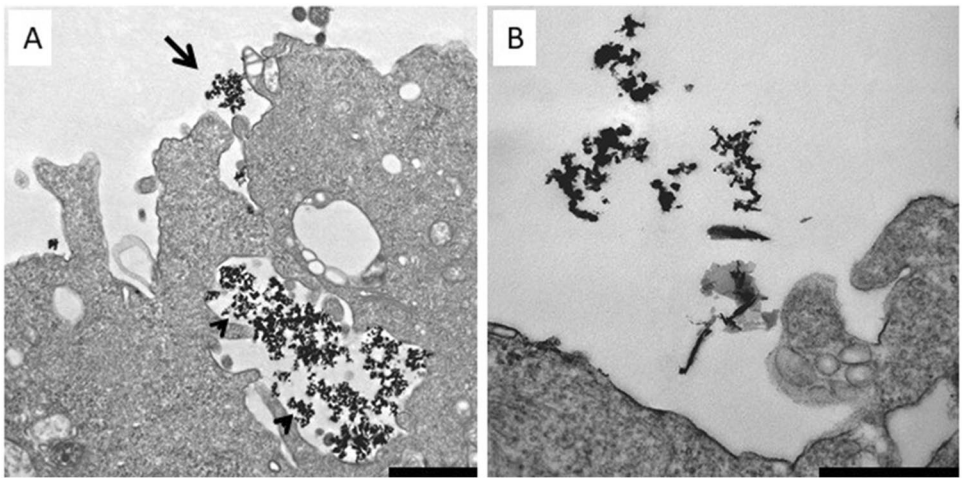

Fig. 7 Transmission Electron Microscope analysis of DLEC cells 24 h after TiO_2-NPs and Cd co-exposure. A. TiO_2-NPs agglomerates in the extracellular area (arrow) and inside the cells (arrowheads). B. Higher magnification of extracellular NPs. Bars: A: 2 µm; B. 500 nm.

longer in nanometre form and no chemically active (Balbi et al. 2014; Nigro et al. 2015; Santonastaso et al. 2020; Yang et al. 2012; Ahamed et al. 2020). In this context, agglomeration of Degussa TiO_2-NPs in different media evaluated by Dynamic Light Scattering (DLS) was already reported (Della Torre et al. 2015). In particular, DLS analysis of TiO_2-NPs suspension with and without Cd revealed that TiO_2-NPs (10 mg/L) agglomerated in artificial seawater (ASW) within 1 min after sonication, leading to marked sedimentation and consequently to a 50–80% reduction of concentration after 6 h (Della Torre et al. 2015). DLS analysis confirmed the formation of agglomerates of TiO_2-NPs of micrometric size within the first minute after sonication, with values of polydispersity index ranging from 0.295 (±0.028) to 0.383 (±0.041) and Z-average ranging from 972 (±35.37) to 1448 (±25.72) nm (Della Torre et al. 2015).

Indeed, a *Trojan horse* effect between TiO_2-NPs and Cd was not found in several experimental models, as the co-exposure resulted in reduced apoptosis, reduced DNA fragmentation, low percentage of intracellular ROS and greater genomic stability due to the aggregation of the two substances (Nigro et al. 2015; Santonastaso et al. 2020; Mottola et al. 2020).

In this work, we analyzed the effects of TiO_2-NPs and Cd interaction in DLEC cell lines in order to verify changes in genotoxicity generated by the co-exposure *in vitro* respect to single exposures in a *vitro* aquatic model.

The results showed that TiO_2-NPs exposure in DLEC induced DNA strand breaks and apoptosis only at the longest exposure time (48 h). These effects might depend both on the TiO_2-NPs form and on the exposure time. We used a TiO_2-NPs formulation consisting of 75% rutile and 25% anatase. It was shown that TiO_2-NPs in the anatase form was absorbed by MeT-5A cells and promoted the generation of intracellular ROS, while the rutile form was mostly unabsorbed (Hattori et al. 2017). However, a previous study demonstrates the ability of TiO_2-NPs with 25% of anatase to penetrate into DLEC cells in 24h exposure without inducing DNA damage (Picchietti et al.2017). In the present study, RAPD-PCR showed that TiO_2-NPs 25% of anatase induced changes in the DLEC genome already at short exposure times, with a reduction in genomic stability for all exposure hours, which was not accompanied by apoptosis and DNA strand breaks after the first two exposure times (3 and 24 h). This result is explained because RAPD-PCR technique is capable of detecting instantaneous changes in the genome, which, if promptly repaired, do not necessarily lead to apoptosis.

Cd has shown genotoxic effects since the shortest exposure, with phenomena of DNA integrity loss, apoptosis and genomic instability due to ROS generation by lipid peroxidation (Picchietti et al. 2017).

Anyway, after the co-exposure of Cd combined with TiO_2-NPs, no genotoxic effect for any tested exposure time was observed. In fact, DLEC co-exposed to TiO_2-NPs and Cd never undergo apoptotic processes and DNA-induced mutations, rather they retain a genomic template stability comparable to the untreated control, unlike DLEC exposed to Cd alone. Concerning TEM in DLEC observations no nanoparticles were found inside the nucleus, indicating the occurrence of indirect mechanisms of genotoxicity.

However, our study is limited to an *in vitro* aquatic model, so it will be necessary to evaluate the bioavailability of the agents tested in an *in vivo* system in seawater (environment where D. *labrax* embryos develop in nature) in order to have more realistic exposure scenarios. Furthermore, it will be necessary to evaluate a time dependent effect of the two substances by increasing the treatment times.

Our interesting findings are in contrast with what reported regarding NPs' toxicity but the explanation might be based on the fact that physical and chemical properties of NMs, such as high stability in water, large surface area, porosity and bonding affinity, give NPs the ability to

capture and bind other contaminants, reducing the concentration of toxic substances in their simple form.

Previous characterization and analytical determinations by UV-Vis spectroscopy highlighted the adsorption of Cd to TiO_2-NPs, which reduced the levels of free NPs compared to their nominal added concentration (Santonastaso et al. 2020). Therefore, we can speculate that when TiO_2-NPs come in contact with Cd in different environments, the NPs aggregate and form a no longer genotoxic structure, unable to react with the genetic material. This peculiarity could be exploited to control water pollution. In this regard, new nanomaterials (on/iron oxide, carbon, titanium/titanium oxide, graphene/graphene oxide, metal organic frameworks (MOFs)) have shown the potential to reduce aquatic contamination by heavy metals (Saharan et al. 2014; Mohmood et al. 2013). Therefore, thanks to their mechanical, electrical and magnetic properties, NMs could be an advantageous tool for the remediation of contaminated environments (Maitlo et al. 2019; Frenzilli 2020).

This peculiarity of NPs is already known since several years. Different types of nanoparticles have demonstrated the ability to remove metallic contaminants from aquatic environments (Daraee et al. 2016). Smaller size NP-Fe_3O_4 showed great arsenic removal efficiency. Similarly, γ-Fe_2O_3 nanocomposites revealed adsorption capacity of zinc (Zn), copper (Cu) and chrome (Cr) from wastewater, as well as a wide range of different nanomaterials (Saharan et al. 2014).

However, despite the promising data, it is still difficult to establish the stability and long-term effects of NMs under various environmental conditions.

The use of TiO_2-NPs to clean contaminated environments through nano remediation processes is suggested as a future research direction. Nevertheless, the results of our study confirm that TiO_2-NPs interacts with Cd softening the genotoxicity of single substances in aquatic environment, at least in the present *in vitro* model.

Further studies using different combination of rutile and anatase and different metals to be co-exposed with are needed to confirm if TiO_2-NPs does not act as *Trojan horse* by increasing the toxicity of heavy metals.

Moreover, it must be pointed out that current genetic toxicological tests are unable to fully clarify the DNA damage mechanisms at the molecular, subcellular, cellular, organ, system and organism levels (Turkez ey al. 2017). The discovery of novel biomarkers of genotoxicity through omics-based methods, will provide a more comprehensive assessment of the genotoxic potential of chemicals. Hence, further toxicogenomic and epigenomics studies will be needed for identifying the chemicals molecular response and mechanism, and early predict their apical effect (Wang et al. 2018).

Conclusion

The simultaneous presence of NPs and heavy metals in the aquatic environment can have different effects on organisms depending on their concentration and their possible aggregation. In our study, DLEC co-exposed to 1 µg/mL of TiO_2-NPs plus 0.1 µg/mL of Cd showed a lower genomic damage than those exposed to single substances starting from first exposure treatment hours. Furthermore, considering that the association of TiO_2-NPs and Cd causes a reduction in the free concentration of TiO_2-NPs in the exposure water, it is plausible that the two substances form a single less genotoxic complex. These results can lay the foundations for new methods for the remediation of contaminated water. However, further chemical characterization and bioaccumulation studies are necessary in order to confirm the formation of TiO_2-NPs and Cd aggregates unable to penetrate the nucleus being therefore less genotoxic.

Acknowledgments The authors thank Dr. Anna Rita Taddei, Section of Electron Microscopy, Great Equipment Center, University of Tuscia, Viterbo, Italy, and Dr. Patrizia Guidi, Department of Clinical and Experimental Medicine, University of Pisa, Italy, for their help to performing Transmission Electron Microscopy (TEM) in cell.

Authors' Contributions Filomena Mottola: conceptualization, writing and editing; Marianna Santonastaso, Concetta Iovine, Patrizia Guidi and Viviana Genualdo: methodology; Giada Frenzilli: formal analysis and editing; Simona Picchietti: methodology and review; Lucia Rocco: funding acquisition, project administration, review and editing. All authors have read and agreed to the published version of the manuscript.

Funding This research was partially supported by the Italian Ministry of Research (PRIN 2009FHHP2W)

Data availability All data generated or analyzed during this study are included; any additional information is available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent to publish Not applicable

Competing interests The authors declare that they have no competing interests

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