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ENGINEERED NANOPARTICLES OF TITANIUM DIOXIDE (TIO_2): UPTAKE AND BIOLOGICAL EFFECTS IN A SEA BASS CELL LINE

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LIST OF ABBREVIATIONS: ENPs, Engineered nanoparticles; TiO_2-NPs titanium dioxide nanoparticles; TiO_2, Titanium dioxide; DLEC, sea bass continuous embryonic cell line; Cd, Cadmium Chlorid; EDS, energy-dispersive X-ray; ROS, reactive oxygen species; TEM, Transmission Electron Microscopy; FDA, fluorescein di-acetate; SEM, Scanning Electron Microscopy; HO, Hoechst; Ti, titanium; ATP, intracellular adenosine triphosphate.

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ABSTRACT

With the rapid development of nanotechnology there has been a corresponding increase in the application of titanium dioxide nanoparticles (TiO_2-NPs) in various consumer and industrial products, consequently their potential health hazards and environmental effects are considered an aspect of great concern.

In the present study, in order to assess the impact of TiO_2-NPs in the marine environment, the biological effects of TiO_2-NPs on a sea bass cell line (DLEC) were investigated. Cells were exposed for 24 h to different concentrations of TiO_2-NPs (1, 8, 40, 200 and 1000 μg/ml) or co-exposed with $CdCl_2$ (Cd). The effects of UV light irradiation were also investigated in cells treated with TiO_2-NPs and/or Cd. The internalization of TiO_2-NPs and the morphological cell modifications induced by the treatments were examined by transmission and scanning electron microscopy, this latter coupled with energy dispersive X-ray spectroscopy (EDS) for particle element detection. In addition, the effects of controlled exposures were studied evaluating the cytotoxicity, the DNA damage and the expression of inflammatory genes.

Our study indicates that TiO_2-NPs were localized on the cell surface mainly as agglomerates revealed by EDS analysis and that they were uptaken by the cells inducing morphological changes. Photoactivation of TiO_2-NPs and/or co-exposure with Cd affects ATP levels and it contributes to induce acute cellular toxicity in DLEC cells dependent on Ti concentration. The inflammatory potential and the DNA damage, this latter displayed through a caspase-3 independent apoptotic process, were also demonstrated.

Overall our data suggest that the interaction of TiO_2-NPs with marine water contaminants, such as cadmium, and the UV irradiation, may be an additional threat to marine organisms.

KEYWORDS: TiO_2 nanoparticles, $CdCl_2$, Sea bass, Uptake, Immune system, *In vitro* toxicology

INTRODUCTION

The production of nanomaterials is increasing worldwide. Engineered nanoparticles (ENPs) are used in diverse industrial fields (Dunphy Guzman et al., 2006) and new applications are constantly arising (Wei et al., 2007; Li et al., 2008; Sekhon, 2010; Bradley et al., 2011; Peters et al., 2014; Smolkova et al., 2015). This means that the environmental contamination with ENPs is becoming a major issue (Aitken et al., 2006; Dusinska et al., 2013). In fact, there is a general concern about the potential hazards posed by released ENPs not only toward humans but also with respect to other organisms present in the environment (Kipen and Laskin, 2005; Kagan et al., 2005; Curtis et al., 2006; Hardman, 2006; Heinlaan et al., 2008; Bondarenko et al., 2013; Parivar et al., 2015).

Between these materials, the metal oxide nanoparticulate, and in particular the nanoparticles of titanium dioxide (TiO_2-NPs) are among those produced at the highest volume. TiO_2 is a versatile compound that is used in nano-form in a variety of consumer products, such as sunscreens and other cosmetics (Wakefield et al., 2005), specialist coatings and paints (Guarino et al., 2008; Kandavelu et al., 2004), food-processing technology (Peters et al., 2014) and in industrial photocatalytic processes (Guillard et al., 2003; Zhang et al., 2006). Thus it has the great potential to be released into the aquatic environment, including surface waters that receive industrial and municipal effluents (Pitkethly, 2004; Klaine et al., 2008). Moreover, we have to take into account that ENPs' behavior depends on the composition of water constituents; in fact particles may agglomerate and interact with the organic material and natural colloids present in these systems, which in turn will likely affect the ENPs potential ecotoxicity and their bioavailability to aquatic organisms (Handy et al., 2008; Baun et al., 2008). However, relatively little is known about the magnitude, the fate and behavior of nanoparticles entering into the bodies of aquatic organisms and their subsequent biological effects (Colvin, 2003; Dowling et al., 2004; Oberdörster et al., 2005; Nigro et al., 2015; Della Torre et al., 2015a; Vannuccini et al., 2015) and regard the possible bioaccumulation in species used for human food.

Therefore, considering that the interaction of nanomaterials with cells can be regarded as a first step in the induction of possible health problems, some *in vitro* studies have focused on elucidating the uptake and biological effects of TiO_2-NPs in cell lines, being the *in vitro* systems the best experimental model for studying toxic mechanisms at the molecular and cellular levels in a controlled environment (Castano et al., 2003). Common findings include general cytotoxicity (Soto et al., 2005; Shi et al. 2013), induction of an inflammatory response (Sayes et al., 2006), as well as generation of free radicals (Donaldson et al., 1996), reactive oxygen species (ROS) and oxidative damage (Long et al., 2007; Sayes et al., 2006). The studies have also shown the ability of TiO_2-NPs to cross cell membranes (Geiser et al., 2005) and induce micronuclei formation and apoptosis (Rahman et al., 2002).

In this context, in order to study the intrinsic hazard potential of TiO_2-NPs that may enter into fish from the aquatic environment, different biological effects of NP-TiO_2 on a sea bass continuous embryonic cell line (DLEC) (Buonocore et al., 2006) were investigated. Moreover, as the uptake and localization of nanoparticles are relevant for general cytotoxicity and induction of inflammatory responses, we examined the distribution and internalization of TiO_2-NPs in DLEC cells both by Scanning Electron Microscopy (SEM), coupled with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection, and by Transmission Electron Microscopy (TEM). In addition, as it is well known that the TiO_2-NPs may bind dangerous substances present in traces in marine water such as cadmium (Zhang et al., 2007; Hartmann et al., 2010; Yang et al., 2012), and can absorb UV light (Yin et al., 2012), catalyzing the generation of ROS, such as superoxide anion radicals, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen in aqueous media (Konaka et al., 1999, 2001; Hirakawa et al., 2004; Shi et al., 2013), the effects of controlled TiO_2-NPs exposure and combined treatment with UV light and/or $CdCl_2$ (Cd) were analyzed in term of quantitative parameters related to metabolic functions, morphological modifications, DNA damage and expression of some inflammatory related genes.

2. MATERIALS AND METHODS

2.1. Suspension of TiO_2-NPs

The suspension of the nanosized Titanium Dioxide (TiO_2-NPs), namely Aeroxide® (provided by Eigenmann & Veronelli, Milan, Italy; declared purity: 99.9%), was obtained according to the protocol described by Allouni et al. (2012). Briefly, a stock suspension of 10 mg/ml of TiO_2-NPs, previously characterized by analytical (Della Torre et al., 2015) and morphological techniques (Nigro et al., 2015), was added in FBS-free medium and sonicated for 1 min (VCX130, Vibra-Cell, 130 W, Sonics & Materials Inc., USA). The end-point concentrations of TiO_2-NPs for exposure were 1, 8, 40, 200 and 1000 μg/ml.

2.2 Cell culture and treatments

The DLEC cells, a continuous embryonic cell line established from sea bass (*Dicentrarchus labrax* L.) (Buonocore et al., 2006), were cultured in flasks (BD Falcon, Tissue culture treated, seal cap) at 22°C in Leibovitz L-15 medium (Sigma-Aldrich) supplemented with 1% L-glutamine, 100 U/ml penicillin-streptomycin and 10% FBS. The cells were treated according to the experiment schedule which is designated in Table 1. $CdCl_2$ (Cd) 99% (Sigma-Aldrich) (0.1 μg/ml) nominal concentrations were chosen in accordance with a previous pilot study (Nigro et al., 2015). Differently, the intensity and the time of the UVA light exposition (30,000 $\mu W/cm^2$ for total 24 min.) from five fluorescent 8-Watt UV-A lamps (365 nm) (Spectrolinker™ XL-1000A) were chosen in accordance with results shown in supplemental data section. The samples were analyzed by *ATPlite™* assay, SEM, TEM, SCGE analysis and real time PCR as reported in Table 1. In particular, the study focused on 1 μg/ml TiO_2-NPs: this dose was chosen as it is far below the LC50 reported for fish species but still able to induce significant biological responses (Xiong et al., 2011).

148 *2.3 ATPlite assay*

149 The intracellular adenosine triphosphate (ATP), widely accepted as a valid marker of viable cells,
150 was measured by the *ATPlite™* assay system (Perkin-Elmer), according to the manufacturer's
151 instructions. DLEC cells were transferred (~10,000/well) to polystyrene 96-microwell plates
152 (Perkin-Elmer) and cultured overnight at 22°C in FBS-free L-15 medium, then the treatments were
153 performed for 24 h as reported in Table 1. Controls were operated by changing the medium every
154 two days to cultivate the cells at 22 °C (negative control) or by addition of 0.2% NaN_3 (positive
155 control) for 24 h.
156 The ATP lite assay is based on the production of light caused by the chemical reaction of ATP with
157 added luciferase and D-luciferin. The amount of emitted light, linearly correlated with ATP
158 concentration (Cree et al., 1997), was measured with a luminometer (Victor II Perkin-Elmer) for 10
159 minutes in the dark. Five independent experiments and three replicates per treatment were
160 performed.

161 *2.4 Scanning electron microscopy (SEM) and microanalysis*

162 For SEM analysis, cells (70,000) were seeded on sterile glass coverslips inserted in 24-well cell
163 culture plates (IWAKI, Scitech Div. Asahi Techno Glass). The cells were cultured overnight at 22
164 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). The
165 control was obtained adding fresh FBS-free medium for 24 h.
166 After treatments, the samples were fixed overnight at 4 °C with 2.5% paraformaldehyde and 2.5%
167 glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (all reagents from Sigma-Aldrich). Samples were
168 washed four times with cacodylate buffer, then post-fixed with 1% osmium tetroxide (Sigma-
169 Aldrich) and 0.15% ruthenium red (Sigma-Aldrich) in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4
170 °C. After different washings in distilled water, the samples were dehydrated with a graded acetone
171 series (from 30% to 100%) and then dried with the critical point method, using CO_2 in a Balzers
172 Union CPD 020. Dried coverslips were gold-coated in a sputtering unit equipped with an argon inlet

(Balzer Union MD 010) for the observations by SEM (Jeol JSM 6010LA) (Tokyo, Japan). The identity of putative TiO_2-NPs was confirmed using the SEM (Jeol JSM 6010LA) (Tokyo, Japan) in combination with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection.

2.5 Transmission Electron Microscopy (TEM)

For TEM analysis, cells (40,000) were seeded on sterile PET track-etched membrane (3 µm pore size) that were inserted in 24-well cell culture plates (IWAKI, Scitech Div. Asahi Techno Glass). The cells were cultured overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). The 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 0.1 M cacodylate buffer at pH 7.2 (all reagents from Sigma-Aldrich). Samples were washed four times with cacodylate buffer, then post-fixed with 1% osmium tetroxide (Sigma-Aldrich) and 0.15% ruthenium red (Sigma-Aldrich) in 0.1 M cacodylate buffer at pH 7.2 for 1 h at 4 °C. After different washings in distilled water, the samples were dehydrated with a graded acetone series (from 30% to 100%) and embedded in epon-based resin. The 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

2.6 SCGE analysis (comet assay)

The standard alkaline (pH > 13) single-cell gel electrophoresis (SCGE), or comet assay, was carried out as described earlier under visible fluorescent light (Tice et al., 2000). For the SCGE analysis the cells were cultured in flasks (BD Falcon, 500,000 cells/75cm^2) overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). To include a positive control cells were X-irradiated with 3 Gy at 37°C with a 250 KV and 6 mA with a Gilardoni MGL 200/8 D

X-ray apparatus, at a dose-rate of 60 cGy/min in FBS-free medium. The negative control was obtained adding fresh FBS-free medium for 24 h. All the experiments were performed in triplicate. After the drug treatments, cells were collected and processed for the assay.

As described by Lorenti Garcia et al., (2006), 20 µl of the cell suspension (5×10^5 cells) were mixed with 80 µl of 0.75% low melting-point agarose in phosphate buffered saline (PBS) at 37°C and placed on frosted glass microscope slide previously coated with a layer of 1% normal melting-point agarose in PBS. Two slides for each experimental point were then incubated in a lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, pH 10, with 1% Triton and 10% DMSO freshly added) for 1 day at 4°C. To allow unwinding of DNA, slides were placed on a horizontal electrophoresis unit and incubated for 15 min with an electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH13). Electrophoresis was performed for 20 min at 25V (volt per cm) and 300 mA at 4°C, then the slides were washed in neutralization solution (0.4 M Tris-HCl, pH 7.5) for 5 min and fixed in methanol for 3 min. Slides were stained with ethidium bromide (20 µg/ml) and covered with a coverslip. An automatic image analyser (Comet Assay III, Perceptive Instruments, UK) connected to a fluorescence microscope (Axioskop 2, Zeiss) was used to examine the stained nucleoids. The amount of DNA damage was evaluated using computer-generated tail moment (tm) values and percentages of DNA damage. For each experimental point, 100 cells were scored from two slides for a total of 200 cells.

2.7 Gene expression experiments

The DLEC cells were grown in tissue-culture-treated flasks (BD Falcon, 500,000 cells/75cm^2) with FBS-free L-15 medium. After an overnight incubation at 22 °C, the medium was removed from the flasks and replaced by the FBS-free medium containing the different treatments (Table 1). The cells were exposed for 24 h to different treatments (Table 1) carried out in triplicates at 22 °C. Controls of the experiments were obtained from flasks in which the old medium was replaced by new FBS-

free medium for 24 h. The medium was then aspirated and the lysis buffer was added (RNeasy Mini Kit, Qiagen).

2.7.1 Reverse-transcriptase reactions and real time PCR

The used protocol was similar as previously described (Picchietti et al., 2009). The absence of DNA contamination was checked using, in a PCR reaction, sea bass *β-actin* primers that bracketed an intron (Table 2). For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following manufacturer's instructions.

The transcript levels of caspase-3, *IL-8* and *TGF-β*, and *rRNA-18s* (used as house-keeping gene) were determined with a Mx3000P™ real-time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions. ROX was used as internal passive reference dye since it is not reactive during real-time PCR and therefore can be used to normalize slight differences in the volume of the added real-time PCR reaction mix, transparency of the plastic caps and other sources of well-to-well differences.

Specific PCR primers (Table 2) were designed for the amplification (~200 bp) of the analysed apoptosis and inflammatory-related genes.

In each PCR reaction 10 ng of cDNA template was used. The PCR conditions for caspase-3 were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s. Differently the PCR conditions for IL-8, TGF-β and *rRNA-18s* were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, that was replaced with water in all blank reactions. The analysis was carried out using the endpoints method option of the Mx3000P™ software and the fluorescence was collected at the end of each extension stage of amplification. A relative quantification was performed and the untreated cells were used as calibrator (defined as 1.0) for the

quantification of transcripts of each gene of interest, performed in separate runs. A normaliser target (*rRNA-18s*) was used to correct for differences in total cDNA input and the quantitative assessment was based on determination of threshold cycle.

2.8 Statistical analysis

Numerical data are presented in the text as means ± SD. Homogeneity of variances was tested before data processing. Data from *ATPlite™* assay and from experiments on multiple genes expressions were analysed by one-way ANOVA, followed by Bonferroni's test. Data from SCGE analysis (data with non-normal distribution) were analysed by Kruskal-Wallis test followed by Dunn's Multiple Comparison test. Data were analyzed using the GraphPad Prism 4.0 software statistical package. Numerical values obtained by all the treatments were compared to the control (untreated cells), when treatments with X-ray or UV light irradiation were performed, the numerical data were also compared to UV-irradiated cells. The level for accepted statistical significance was $P<0.05$.

3. RESULTS

3.1. ATP measurements

TiO_2-NPs, dispersed according to the protocol in the M&M section, were investigated for their effect on DLEC intracellular ATP levels. As it is shown in Fig. 1 A, different TiO_2-NPs concentrations (1, 8, 40, 200 and 1000 μg/ml) did not significantly affect the intracellular ATP levels in DLEC cells. However, a significant decrease ($P<0.001$) of ATP level was found in DLEC cells treated with 0.2% NaN_3 (positive control) compared to the negative control.

As it is known that TiO_2-NPs might also interact with other co-existing environmental pollutants (Yang et al., 2012; Hartmann et al., 2010), as metals and organic xenobiotics, ATPlite™ assay was also performed to evaluate the effect of the combined treatment: TiO_2-NPs + Cd (Table 1). As it is shown in Fig. 1 B, DLEC cells treated with TiO_2-NPs (1000 μg/ml) + Cd and Cd alone showed a

274 significant lower intracellular ATP level compared to control, while when cells were treated with
275 lower concentration of TiO_2-NPs + Cd no significant differences were found compared to the
276 control (Fig. 1 B).
277 A previous work demonstrated that TiO_2-NPs can be photoactivated (Yin et al., 2012) and, to study
278 this aspect, DLEC cells were exposed to TiO_2-NPs + UV light (Table 1). The intensity and the time
279 of the UV irradiation, which could be used without inducing significant decrease in intracellular
280 ATP levels, were evaluated by *ATPlite™* assays performed on DLEC cells exposed to increasing
281 doses of UV light with a time of recovery of 24, 48 and 72 h. As the ATP amounts in all the treated
282 and control groups were not significantly affected by the different UV doses, the maximum UV
283 irradiation (30,000 μW/cm^2 for 24 minutes) with a time of recovery of 24 h was chosen for the
284 successive experiments (Figure A, supplementary data section).
285 When the cells were treated with TiO_2-NPs (1 μg/ml) + UV or with TiO_2-NPs (1 μg/ml) + Cd +UV,
286 neither the photoactivation of the nanoparticles nor Cd exposition induced significant changes of
287 the intracellular ATP levels (Fig. 2 A). Differently, the ATP level was significantly affected in cells
288 treated with 1000 μg/ml of photoactivated TiO_2-NPs (Fig. 2 B) when compared to control or UV
289 treatment. The ATP level was also affected in cells treated with 1000 μg/ml of photoactivated TiO_2-
290 NPs+Cd and the decrease was statistically significant. Conversely, the exposure to Cd+UV
291 significantly increased the ATP level in DLEC cells compared to control or UV treatment (Fig. 2
292 B).
293
294 *3.2 Cellular morphological modifications*
295 Morphological modifications were observed in the cells exposed to TiO_2-NPs compared to control
296 (Figs. 3 A-G). The control cells were flat, showing short protrusions on the cell surface (Figs.3 A-
297 B), while cells treated with TiO_2-NPs (1 μg/ml or 1000 μg/ml) showed smoother cell surface and
298 abundant flocculate material on their surface and filopodia (Figs. 3 D, F). In particular, an increase

of cells characterized by a reduction of protrusions density was observed when DLEC cells were treated with increasing concentration of TiO_2-NP (Figs. 3 D, F).

Scanning electron microscopy in combination with energy dispersive X-ray spectroscopy (EDS) revealed agglomerates of particles with strong signals in treated cells (1 μg/ml or 1000 μg/ml TiO_2-NPs) (Figs. 3 E, G), but not in control cells (Fig. 3 C). Elemental analysis of the observed agglomerates by EDS showed an X-ray energy peak belonging exclusively to titanium (Ti) (Fig. 3H).

Cells treated with TiO_2-NPs (1 μg/ml) and Cd showed short protrusions and flocculate material on their surface (Fig. 4 A) with strong EDS signals. Round shape and membrane budding were also observed in some cells (data not shown). Differently, the majority of the cells treated with TiO_2-NPs (1000 μg/ml) and Cd appeared destroyed (Fig. 4 B) and showed aggregates of Ti identified by EDS. Morphological modifications were found in cells treated with Cd alone (Fig. 4 C) showing longer protrusions than control cells (Figs. 3 A, B). EDS spectra did not reveal any Ti peak in Cd treated cells as expected.

Cells exposed only to UV showed fusiform shape, and numerous protrusions on the surface (Fig. 4 D). EDS spectra did not reveal any Ti signal as expected. Differently, fusiform cells showing abundant Ti flocculate material on their surface, together with cells having rounded shape and membrane budding were observed after the treatment with TiO_2-NPs (1 μg/ml) photoactivated by UV light (Figs. 4 E, F). Cells treated with TiO_2-NPs (1000 μg/ml) photoactivated by UV light appeared destroyed (Fig. 4 G). In this case elemental map analysis showed Ti signals on the surface. Fusiform and round shaped cells with membrane budding were observed after TiO_2-NPs (1 μg/ml) photoactivated by UV light and Cd treatment (Fig. 4 H). In addition, flocculate material identified as Ti by EDS analysis was found on the cell surface. Cells treated with TiO_2-NPs (1000 μg/ml) photoactivated by UV light and Cd appeared destroyed (Fig. 4 I) and covered by flocculate Ti

material. Cell treated with Cd + UV showed fusiform shape and protrusions on the surface; EDS spectra did not reveal any Ti peak as expected.

3.3 Uptake of TiO_2 nanoparticles.

TEM analysis of DLEC cells exposed to TiO_2-NPs for 24 h (Table 1) pointed out the presence of agglomerates of nanoparticles on the cell surface that were uptaken by the cells (Figs. 5 A-C). At this time, the incorporation of TiO_2-NPs aggregates in the cell was evident (Figs. 5 A-B), although small aggregates were rarely found in "coated" pits (Fig. 5 A). Internalized TiO_2-NPs aggregates (Figs. 5 B-C) were mostly trapped in vesicles that were distributed across the cytoplasm and in the perinuclear region of nucleus (Fig. 5 C). Control cells did not show any NPs (Fig. 5 D).

When cells were treated with both TiO_2-NPs (1 μg/ml) and Cd for 24 h (Table 1), the areas around the cells were provided with TiO_2-NPs agglomerates and, in addition, TiO_2-NPs appeared in vesicles, disseminated in the cytoplasm (Fig. 5 E). Apparently the Cd treatment did not modify TiO_2-NPs uptake, although portions of the cell membranes were damaged by the treatment (Fig. 5 E). Cells treated with Cd showed plasma membrane integrity and absence of TiO_2-NPs in the cytoplasm (Fig. 5 F). The photoactivation of TiO_2-NPs did not interfere with their uptake by the DLEC cells, while absence of TiO_2-NPs was confirmed in Cd + UV and Cd only treated cells (data not shown).

3.4 DNA damage

The amount of DNA damage after the different treatments was quantified by comet assay (Fig. 6).

In particular, a significant increase of DNA damage compared to control and UV light was found after TiO_2-NPs + Cd + UV and Cd + UV treatments. Similarly, the damage significantly increased in cells treated with Cd alone when compared to control. Cells treated with X-Ray showed a significant higher amount of DNA damage compared to control or UV.

348 *3.5 mRNA expression of apoptosis and inflammation-related genes*

349 A specific amplification of the expected product related to caspase-3 was not found both in DLEC
350 cells exposed to the different treatments and controls. However, a specific amplicon, used the same
351 primers, was identified in the thymocytes of a sea bass juvenile (data not shown), as the caspase-3
352 activation is an essential protein in the apoptosis process.

353 The expression of the inflammation-related genes such as IL-8 and TGF-β (Figs. 7 A-D), was
354 quantified in DLEC cells after 24 h of treatments (Table 1).

355 A significant up-regulation of the IL-8 transcripts compared to the control was quantified in DLEC
356 cells treated with TiO_2-NPs or TiO_2-NPs + Cd, while a significant down-regulation was found after
357 Cd treatment (Fig. 7 A). After photoactivation, the levels of IL-8 transcripts in cells treated with
358 TiO_2-NPs + UV, TiO_2-NPs + UV + Cd or Cd + UV were significantly different compared to UV.
359 The irradiation of the cells with UV light alone significantly reduced the IL-8 transcripts compared
360 to the control (Fig. 7 B).

361 The TGF-β levels significantly increased in cells treated with TiO_2-NPs being higher compared to
362 the control (Fig. 7 C). In addition, the level of the TGF-β transcripts after Cd treatment was
363 significantly higher than the control (Fig. 7 C).

364 After the photoactivation, the levels of TGF-β transcripts (Fig. 7 D) were significantly up-regulated
365 in cells treated with TiO_2-NPs + UV + Cd compared both to the control and UV, while in TiO_2-
366 NPs+UV treated cells the TGF-β levels were down-regulated compared to the control but they were
367 significantly higher compared to UV. Cd + UV treatment significantly increased the TGF-β
368 transcripts when compared to UV. A down regulation of TGF-β transcripts was also found after UV
369 treatment when compared to the control (Fig. 7 D).

370

371

372

DISCUSSION

The present study investigates the TiO_2-NPs induced toxicity in a teleost cell line (Buonocore et al., 2006). In addition, it evaluates the capability of TiO_2-NPs to form aggregates and cross the membranes inducing cell morphological changes, DNA damage and inflammatory responses.

TiO_2-NPs photoactivated by UV and Cd co-exposure affect ATP levels in DLEC cells depending on TiO_2 concentration. The *ATPlite™* assay revealed that the viability of the DLEC cells was not significantly affected by the different concentration of TiO_2-NPs (1, 8, 40, 200, 1000 μg/ml), although the compound exhibited a biphasic dose-response curve characteristic of hormesis-like effects (Mattson 2008). The treatment with Cd (0.1 μg/ml), a common aquatic toxic metal pollutant (Schultz et al., 1996; Iliopoulou-Georgudaki and Kotsanis, 2001), which is known for its ability to affect essential cellular processes such as cell division, proliferation, differentiation and apoptosis (Petanidis et al., 2013; Morcillo et al., 2016 a,b), significantly reduced the ATP level compared to control. Differently, the co-exposure of TiO_2-NPs with Cd resulted in increased adverse effects dependent on TiO_2 concentration.

To understand these results, it is important to consider that the uptake/accumulation of Cd in DLEC cells, and consequently its toxicity, might be affected by the presence of TiO_2-NPs. Studies on different freshwater model species highlighted the interference of metals in NPs uptake and vice versa (Tan and Wang 2014; Rosenfeldt et al., 2014; Pavagadhi et al., 2014) and, in a recent paper, Della Torre et al., (2015b) demonstrated that the co-exposure of Cd with TiO_2-NPs did not increase, but rather decrease, the Cd content in *Mytilus galloprovincialis* gill cells. The authors suggested that this observation, together with the results on heavy metal detoxification response (metallothionein induction) could indicate the absence of a Trojan horse effect of TiO_2-NPs toward $CdCl_2$, confirming previous data (Balbi et al., 2014).

After photoactivation, the toxicity of TiO_2-NPs in DLEC cells was dependent on Ti concentration, in fact the ATP level was significantly affected only in cells treated with 1000 μg/ml TiO_2-NPs +

399 UV. Previous studies reported that nanosized TiO_2 alone (0.1-1000 μg/ml) had little effect on gold
400 fish skin cells, whereas co-exposure with UV caused a significant decrease of cell viability
401 dependent on both the concentration of TiO_2 and the dose of administrated UV (Reeves et al.,
402 2008). Similarly, the photoactivation and the co-exposure with Cd induced in DLEC cells a TiO_2-NPs
403 dose-dependent decrease of the intracellular ATP levels compared to control.

404
405

406 **TiO_2-NPs induce cellular modifications.** By SEM analysis, it was evident that TiO_2-NPs (1 μg/ml
407 and 1000 μg/ml) induced cellular modifications, that need to be considered as a sign of cellular
408 toxicity (Ryabchikova et al., 2010; Allouni et al., 2012). In particular, TiO_2-NP dose-dependent
409 reduction of cellular protrusions was found in DLEC cells suggesting that nanoparticles could affect
410 fundamental processes of the cells, including cell migration and invasion as previously shown in
411 human epithelial intestinal cells exposed to TiO_2-NPs (Koeneman et al., 2010; Gagliardi et al.,
412 2015). In human keratinocytes (Fujita et al., 2009), it was demonstrated that TiO_2-NPs can affect
413 extracellular matrix components whose interactions with the cell, mediated by adhesion molecules,
414 are important for the regulation of the cellular shape and for the maintenance of activities such as
415 cell migration, growth, and differentiation (Steffensen et al., 2001). These considerations support
416 the idea that the cellular toxicokinetic of nanoparticles could be focused both on intracellular and
417 extracellular distribution (Hardman 2006). The irradiation of TiO_2-NPs with UV light further
418 induced cellular modifications. Cells treated with 1 μg/ml TiO_2-NPs irradiated with UV showed
419 plasma membrane blebbing, which is a characteristic of the apoptotic process. Similarly, the co-
420 exposure of TiO_2-NPs with Cd, as previously reported in other cell lines (Goering et al., 1995;
421 Thophon et al., 2003; Giari et al., 2007), and the exposure to TiO_2-NPs + UV + Cd induced
422 morphological modifications, like rounded shape and plasma membrane blebbing, suggesting that
423 the photoactivation and the combined exposure with Cd could promote significant rearrangement of
424 the cytoskeleton leading to increased hydrostatic pressure and subsequent detachment of the

membrane from the cortex (Charras 2008). Longer protrusions compared to control were found in Cd treated cells confirming that Cd induced cellular alterations in DLEC cells as previously showed in different sea bass cell types (Giari et al., 2007). When the cells were treated with 1000 µg/ml TiO_2-NPs co-exposed with Cd and /or irradiated with UV light, the toxic effect of the treatments was revealed by the evident morphological damage of the majority of the cells. Such features should be considered as signs of an autolytic necrotic outcome of a complete cellular death program (Jezek and Hlavata, 2005; Valko et al., 2006), when scavengers do not operate. This autolysis was called secondary necrosis by Wyllie et al. (1980) intending to distinguish this type of cell elimination from ‘‘cellular necrosis occurring ab initio”, which should be called ‘‘primary necrosis” (Leist et al., 1997; Kroemer et al., 1998; Nicotera et al., 1998; Golstein and Kroemer 2007).

TiO_2-NPs penetrate into the cell via surface folds and invaginations.

The events start with the direct contact of TiO_2-NPs with the cell membrane that is suggestive of possible macromolecule internalization mechanisms induced by the nanoparticles (Chithrani et al., 2006; Farré et al., 2009). Few cases of TiO_2-NPs in “coated” pits that could represent the initial stages of clathrin-dependent receptor-mediated endocytosis finishing in endosomes (Parkar et al., 2009; Jovic et al., 2010) were found in DLEC cells. However, the amount of the “coated” pits was very small suggesting that this mechanism is only occasionally used and that receptor-mediated endocytosis does not provide appreciable internalization of TiO_2-NPs. Our electron microscopic examinations showed formation of small electron dense agglomerations, direct contact of these latter with the cell plasma membrane and successive penetration into the cell via surface folds and invaginations. This could be due to an increase of the fluidity of cell plasma membrane, allowing it to readily form deep invaginations and providing penetration of the nanoparticles inside the cell. The ability of TiO_2-NPs to destabilize cell membranes after *in vitro* exposure of cell lines has also been noted by other authors (Simon-Deckers et al., 2008; Thevenot et al., 2008; Lee et al., 2009). Moreover, it should be considered that TiO_2-NPs generate free radicals, including oxygenated free

radicals and carbon-centered radicals, causing cleavage of C-H bonds in organic molecules that could be the first step of oxidative damage of biological molecules (Chen and Mao, 2007; Fenoglio et al., 2009). It has been reported that lipid peroxidation may result from interactions between nanoparticles and lipids (Kiwi and Nadtochenko, 2004) or from elevated concentrations of ROS which are capable of peroxidising membranes (Sayes et al., 2005) with consequent destabilization (Gurr et al.,2005; Wang et al., 2009). After 24 h of exposure TiO_2-NPs seemed to penetrate into the cytoplasm and locate in the peri-region of the nucleus as aggregated particles, which may induce direct interactions between the particles and cellular components, to cause adverse biological responses, as previously reported in cultured human-derived retinal pigment epithelial cells (ARPE-19) after exposure to TiO_2-NPs (Zucker et al., 2010) and in L929 fibroblasts (Allouni et al., 2012). The role of Cd in cellular uptake of nanoparticles was also investigated, revealing that it not interfere with the internalization process. However, portions of the cell membranes were damaged by the treatment, probably due to Cd that, as reported by several previous reports, could cause lipid peroxidation (Ikediobi et al., 2004; Yadav and Khandelwal, 2006; Newairy et al., 2007).

Photoactivation of TiO_2-NPs and co-exposure with Cd contribute to strengthen the genotoxic effect in DLEC cells. It is known that TiO_2 absorbs UV light, catalyzing the generation of ROS (Hirakawa et al., 2004; Konaka et al., 1999, 2001; Kockler et al., 2014), important factors in the apoptotic process, whose excess induces mitochondrial membrane permeability and damages to the respiratory chain, triggering the apoptotic process (Jezek and Hlavata, 2005; Valko et al., 2006). In DLEC cells the photoactivation of the TiO_2-NPs when co-exposed to Cd contributes to strengthen the genotoxic effect. DNA damage was revealed with the appearance of a prominent comet tail due to irreparable double strand breaks (Cimpan et al., 2000; Lukandu et al., 2009). To notice, Cd + UV treatments significantly increased DNA damage in DLEC cells compared to control or UV treatment, although did not induce a toxic effect on cell viability (ATPlite assay). These results confirmed previous data showing in CHO cells that non-toxic concentrations of cadmium affected

the repair of UV-induced DNA damage, providing evidence that the inhibition of DNA repair is an important mechanism of Cd induced mutagenicity and carcinogenicity (Hartwig and Beyersmann, 1989; Fatur et al., 2003). Similarly, Cd alone induced a significant increase of DNA strand breaks in DLEC cells compared to controls. Cd is also known to induce oxidative stress by depletion of glutathione in association with mitochondrial damage, induction of apoptosis and disruption of calcium signaling (Emmanouil et al., 2007; Thévenod, 2009; Vincent-Hubert et al., 2011).

The ineffectiveness of 1 μg/ml nano-TiO_2 and TiO_2-NPs + UV exposures in term of DNA primary damage induction in DLEC cells suggests that different cell types with various activities could exhibit differential genotoxic responses dependent on TiO_2-NPs concentration. Previous studies demonstrated that much higher TiO_2-NPs concentrations than the ones used in our comet assays resulted in micronuclei formation and apoptosis in Syrian hamster embryo fibroblasts (Rahman et al., 2002), human astrocytes-like astrocytoma U87 cell, normal human fibroblasts (Lai et al. 2008) goldfish skin (GFSk-S1), rainbow trout gonadal tissue (RTG-2) and rat kidney proximal (NRK-52E) cells (Reeves et al., 2008; Barillet et al., 2010).

To notice that in samples co-exposed with TiO_2-NPs and Cd the DNA damage was not different compared to control suggesting that TiO_2-NPs might act as an antagonist like previously reported in *Mytilus galloprovincialis* gill cells (Della Torre et al., 2015b).

Caspase-3 is not an integral part of the apoptotic response induced by TiO_2-NPs both under UV or Cd exposure. To elucidate the mechanisms of cell death induced by TiO_2-NPs, the signaling pathways involved in apoptosis were also investigated. It is well known that caspase-3 is one of the main executioner caspases in the apoptotic pathway, cleaving and inactivating a number of molecules and largely contributing to the apoptotic phenotype and the dismantling of the apoptotic cells. Studies involving caspase-3 knockout mice have described that the presence of caspase-3 is essential for chromatin condensation and DNA degradation in apoptosis, although is not required for γ-irradiation-induced apoptosis in mouse embryonic stem cells (Woo et al., 1998). In fish,

503 information on the apoptotic process is relatively scarce and caspase-3 genes only recently started to
504 be sequenced in zebrafish (Yabu et al., 2001), rainbow trout (Rojas et al., 2012) and in sea bass
505 (Nascimento et al., 2007). Interestingly, in DLEC cells caspase-3 specific transcripts were not found
506 suggesting that the apoptotic response induced by TiO_2-NPs, both under UV or Cd exposure, could
507 be caspase-3 indipendent. In this regard, Trouiller et al. (2009) suggested that TiO_2-NPs might
508 damage DNA through a direct chemical interaction with the DNA phosphate group or indirectly via
509 oxidative stress and/or inflammatory responses. As in our observations TiO_2-NPs were never found
510 inside the nucleus, it seems probable that TiO_2-NPs could cause DNA damage indirectly through
511 generation of ROS (Jezek and Hlavata, 2005; Valko et al., 2006; Federici et al., 2007; Kang et al.,
512 2008; Reeves et al., 2008) and/or inflammatory processes (Chen et al., 2006; Grassian et al., 2007).
513
514 **Inflammatory potential of TiO_2-NPs**. Whether or not TiO_2-NPs would induce inflammation is a
515 controversial issue. In a *in vitro* study, Petkovic et al. (2011) suggested that TiO_2-NPs anatase was a
516 stronger inducer of intracellular ROS and that, by the induction of ROS, the expression of
517 inflammation-related genes was also increased in a concentration-dependent manner. In our study
518 the expression of inflammation-related genes such as IL-8 and TGF-β was up-regulated in 1 μg/ml
519 TiO_2-NPs treated cells revealing the inflammatory potential of TiO_2-NPs, as previously showed by
520 Chen et al. (2006) in the absence of UV irradiation. Recent studies, reported that TiO_2-NPs in
521 human lymphocytes influenced the expression of genes that encode biomarkers of inflammation,
522 such as IL-8 (Baranova et al., 2015), and that they were potent inducers of TGF-β expression in
523 human pulmonary fibroblasts, partly via an IL-1β-dependent mechanism (Armand et al., 2013). In
524 our model the co-exposure of TiO_2-NPs with Cd up-regulated the IL-8 transcripts, while TGF-β
525 levels were not affected. In addition, the expression of IL-8 decreased while, on the contrary,
526 TGF-β increased after the treatment with Cd alone. These data suggest that Cd may modulate the
527 immune responses as previously demonstrated (Krocova et al., 2000; Leffel et al., 2003).. It should

be noted that in cells irradiated with UV light alone the IL-8 and TGF-β m-RNA expression was significantly down regulated confirming the potentially harmful impact of UV radiation on fish immune functions (Salo et al., 2000; Jokinen et al., 2001, Markkula et al., 2005). UV light is known for its immunosuppressive properties, which are demonstrated by the inhibition of cellular immune reactions and by the exacerbation of infectious diseases (Kripke 1990; Chapmanet al., 1995). UV light induces the release of immunosuppressive cytokines (Schwarz et al., 1993), but obviously can also interfere with the biological effects of cytokines. The photoactivation of TiO_2-NPs co-exposed with Cd modulated the IL-8 and TGF-β levels showing effects that are not easy to understand without further studies.

Conclusions. Our study demonstrated that TiO_2-NPs contact and cross the membranes of DLEC cells. Moreover, the irradiation of TiO_2-NPs with UV light and the co-exposure with Cd contribute to induce morphological changes, acute cellular toxicity, up-regulation of inflammatory related genes and DNA damage through a caspase-3 independent apoptotic process. These results suggest that the interaction of TiO_2-NPs with marine water contaminants, such as cadmium, triggered by the UV irradiation, need to be taken into consideration as potentially harmful to marine organisms.

CONFLICT OF INTERESTS

The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. **ATP measurements of DLEC cells exposed to different concentration of TiO_2-NPs and TiO_2-NPs plus Cd**. ***A.*** Viability (ATP content) of DLEC cells exposed to TiO_2-NPs (1, 8, 40, 200 and 1000 μg/ml) and 0.2% NaN_3 (positive control) for 24 h at 22°C, referred as percent values with respect to untreated control cells. ***B.*** Viability (ATP content) of DLEC cells exposed to TiO_2-NPs (from 1μg/ml to 1000 μg/ml) plus Cd (0.1 μg/ml) for 24 h at 22°C, referred as percent values with respect to untreated control cells. The % of viability is expressed as the mean ± SD from 5 independent experiments (three replicates per treatment). Significantly different from Control: *P<0.05; ***P<0.001.

Figure 2. ATP measurements after TiO_2-NPs photoactivation. ***A.*** Viability (ATP content) of DLEC cells exposed to UV (30.000 μW/cm^2 for total 24 minutes), TiO_2-NPs (1μg/ml)+UV, TiO_2-NPs (1μg/ml) +UV +Cd (0.1μg/ml) or exposed to UV+Cd (0.1μg/ml), referred as percent values with respect to control cells. ***B.*** Viability (ATP content) of DLEC cells exposed to UV (30.000 μW/cm^2 for total 24 minutes), TiO_2-NPs (1000 μg/ml)+UV, TiO_2-NPs (1000 μg/ml) + UV+Cd (0.1μg/ml) or exposed to UV+Cd (0.1μg/ml), referred as percent values with respect to control cells. The % of viability is expressed as the mean ± SD from 5 independent experiments (three replicates per treatment). Significantly different from Control: *** (P<0.001); significantly different from UV: ¨P<0.01; ¨¨P<0.001.

Figure 3. **Morphological modifications and microanalysis of DLEC cells 24 h after the TiO_2-NPs addition.** ***A.*** SEM analysis of DLEC control cells. ***B.*** Higher magnification showing the DLEC cellular surface. ***C.*** Absence of EDS signals in DLEC control cells. ***D.*** TiO_2-NPs (1μg/ml) treated cell showing smoother surface and abundant flocculate material. ***E.*** EDS signal in treated cells

(1μg/ml TiO_2-NPs). ***F.*** TiO_2-NPs (1000 μg/ml) treated cells showing abundant flocculate material on their smooth surface. ***G.*** EDS signal in treated cells (1000 μg/ml TiO_2-NPs). ***H.*** EDS analysis showing an X-ray energy peak belonging to titanium (Ti) in TiO_2-NPs treated cells. Bars: **A**: 10; μm **B**: 1 μm; **D**: 5; μm **F**: 10 μm.

Figure 4. **Morphological modifications and microanalysis of DLEC cells 24 h after TiO_2-NPs+Cd and TiO_2-NPs+UV+Cd treatments.** ***A.*** DLEC cells treated with TiO_2-NPs (1μg/ml) and Cd showing short protrusions and flocculate material on their surface. ***B.*** Destroyed cells after TiO_2-NPs (1000μg/ml) and Cd treatment. ***C.*** Cells treated with Cd alone showing protrusions of the cell membrane. ***D.*** Cells exposed only to UV showing fusiform shape, and numerous botton-shaped protrusions of the cell membrane. ***E.*** TiO_2-NPs+UV treated cell showing fusiform shape and abundant flocculate material on the surface. ***F.*** Membrane budding observed after the treatment with TiO_2-NPs (1μg/ml) phoactivated by UV light. ***G.*** Destroyed cells after the treatment with TiO_2-NPs (1000μg/ml) phoactivated by UV light. ***H***. Fusiform and rounded DLEC cells showing flocculate material after TiO_2-NPs (1μg/ml) +UV+Cd treatment. ***I***. Cell showing destroyed morphology after TiO_2-NPs (1000μg/ml) +UV+Cd treatment. Bars: **A**: 2 μm; **B**: 10 μm; **C**: 5 μm; **D**: 5 μm; **E**: 2 μm; **F**: 2 μm; **G**: 10 μm; **H**: 5 μm; **I**: 10 μm.

Figure 5. **TEM analysis of DLEC cells 24 h after the TiO_2-NPs and TiO_2-NPs plus Cd treatment.** ***A.*** Agglomerates of TiO_2-NPs (1μg/ml) internalized by cells and coated pits (arrow). ***B.*** Cells leading to the incorporation of aggregates inside vesicles. ***C.*** TiO_2-NPs aggregates in vesicles localized in the perinuclear region***.*** ***D.*** Untreated cells. ***E.*** Portions of the cell membrane damaged by the treatment with TiO_2-NPs (1μg/ml) +Cd (arrows) and TiO_2-NPs in vesicles dispersed in the

1083 cytoplasm. ***F***. Plasma membrane integrity in Cd treated cells. Bars: **A**: 1 μm; **B**: 10 μm; **C**: 1 μm;
1084 **D**: 1 μm; **E**: 1 μm; **F**: 1 μm.

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1086 **Figure 6. DNA damage quantified by SCGE analysis (comet assay) after 24 h of treatments. *A.***
1087 For the SCGE analysis the cells were exposed to: TiO_2-NPs (1μg/ml); Cd (0.1μg/ml); UV (six
1088 doses of 5000 μW/cm^2 for total 24 minutes) with a recovery of 24 h; TiO_2-NPs (1 μg/ml) + Cd
1089 (0.1μg/ml); only Cd (0.1μg/ml); TiO_2-NPs (1 μg/ml) + UV; TiO_2-NPs (1 μg/ml)+Cd (0.1μg/ml) +
1090 UV. The control was obtained adding fresh FBS-free medium for 24 h. ***B.*** Untreated cell ***C.*** X-Ray
1091 treated cell (positive control). Significantly different from Control: *P<0.05, ***P<0.001;
1092 significantly different from UV: ˙P<0.05, ¨˙P<0.001.

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1094 **Figure 7. Q-PCR analysis of inflammation-related genes. *A.*** Expression of IL-8 quantified after
1095 the exposure of the cells to different treatments: TiO_2-NPs; TiO_2-NPs+Cd; Cd. Control obtained
1096 adding fresh FBS-free medium to not treated DLEC cells. ***B.*** Expression level of IL-8 quantified
1097 after the exposure of the cells to different treatments: UV**;** TiO_2-NPs +UV; TiO_2-NPs+UV+Cd; Cd
1098 +UV. Control as above. ***C.*** Expression level of TGF-β quantified after the exposure of the cells to:
1099 TiO_2-NPs; TiO_2-NPs+Cd; Cd. Control as above ***D.*** Expression level of TGF-β quantified after the
1100 exposure of the cells to: UV**;** TiO_2-NPs +UV; TiO_2-NPs+UV+Cd; Cd+UV. Control as above.
1101 Significantly different from Control: * P<0.05, ** P<0.01, *** P<0.001; significantly different
1102 from UV: ˙P<0.05, ¨˙P<0.001.

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Table 1. Experiment schedule.

TiO_2-NPs µg/ml	$CdCl_2$ (Cd) µg/ml	UV µWatt/cm^2						
1	_	_	TiO_2	ATP*lite*	SEM	TEM	SCGE	RT-PCR
1	0.1	_	TiO_2+Cd					
_	0.1	_	Cd					
1	_	30,000	TiO_2+UV					
1	0.1	30,000	TiO_2+Cd+UV					
_	_	30,000	UV					
_	0.1	30,000	Cd+UV					
8	_	_	TiO_2					
8	0.1	_	TiO_2+Cd					
40	_	_	TiO_2					
40	0.1	_	TiO_2+Cd					
200	_	_	TiO_2					
200	0.1	_	TiO_2+Cd					
1000	_	_	TiO_2		SEM			
1000	0.1	_	TiO_2+Cd					
1000	_	30,000	TiO_2+UV					
1000	0.1	30,000	TiO_2+Cd+UV					

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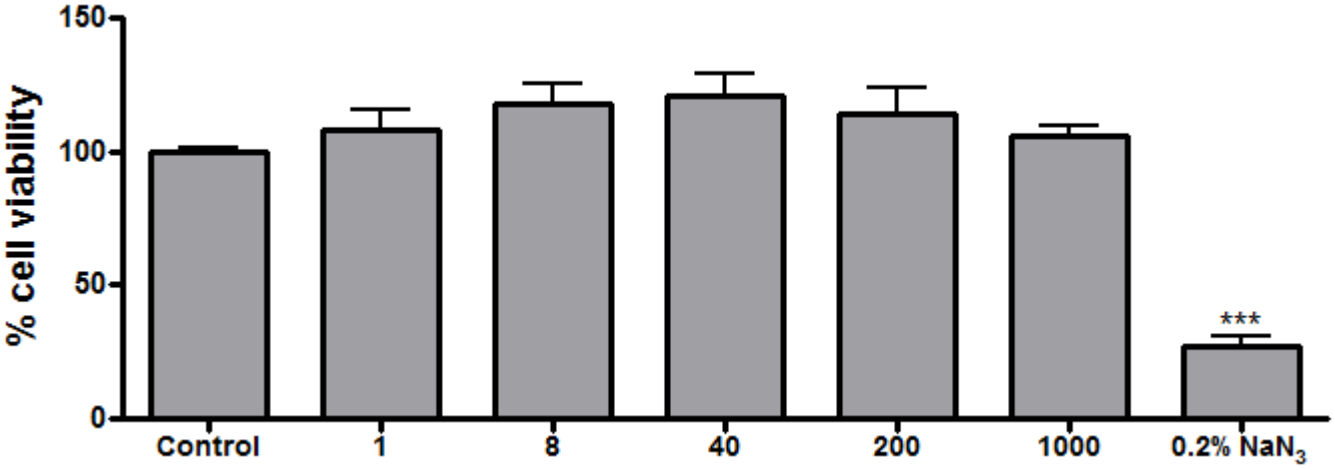
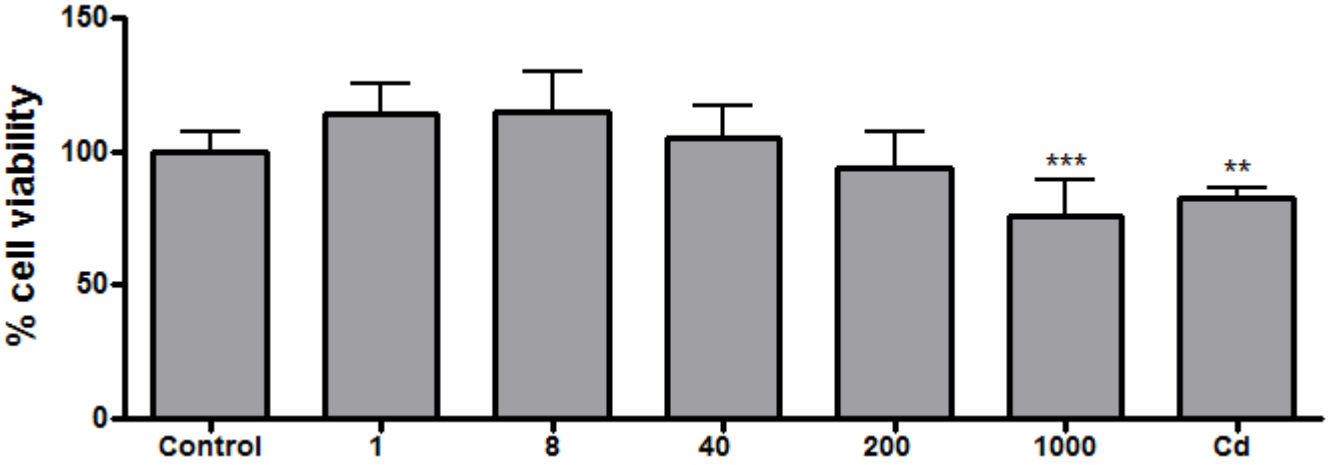
Gene name	***Accession number***	***Primers Sequence (Forward and reverse, 5' → 3')***	***Size (bp)***	***Annealing Temperature (°C)***
Beta-actin	AJ493428	ATGTACGTTGCCATCC GAGATGCCACGCTCTC	550	55
18S Ribosomal RNA	AY831388	CCAACGAGCTGCTGACC CCGTTACCCGTGGTCC	208	52
IL-8	KM225777	GTGCTCCTGGCGTTC CTTCACCCAGGGAGC	205	52
TGF-beta	AM421619	GACCTGGGATGGAAGTGG CAGCTGCTCCACCTTGTG	216	52
Caspase-3	DQ345773	CGACGGACAAGAGTCGGAG CATCGCGTTGCCAGCATCC	223	52

Table 2. Primers for Real time PCR analysis


% cell viability
150
100
50
0
***
Control
1
8
40
200
1000
0.2% NaN3

A)


% cell viability
150
100
50
0
***
**
Control
1
8
40
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1000
Cd

B)

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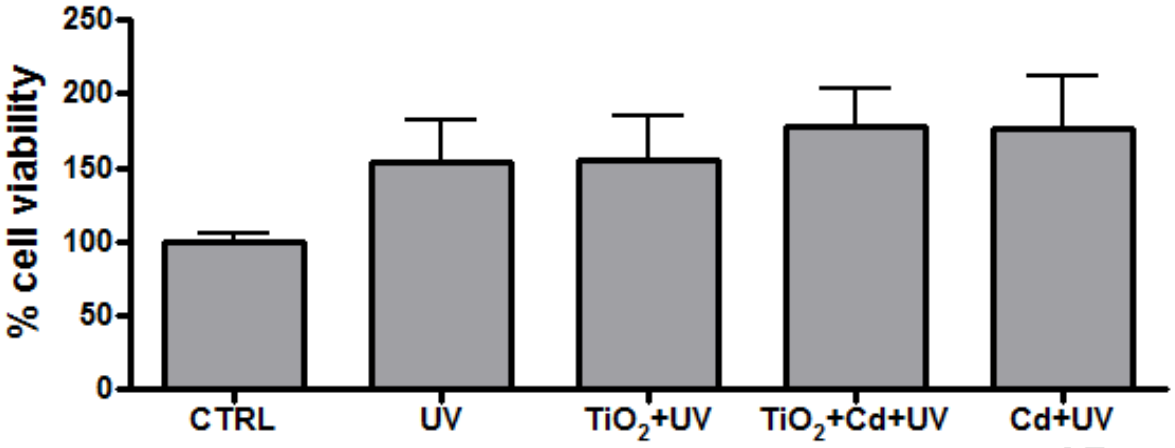
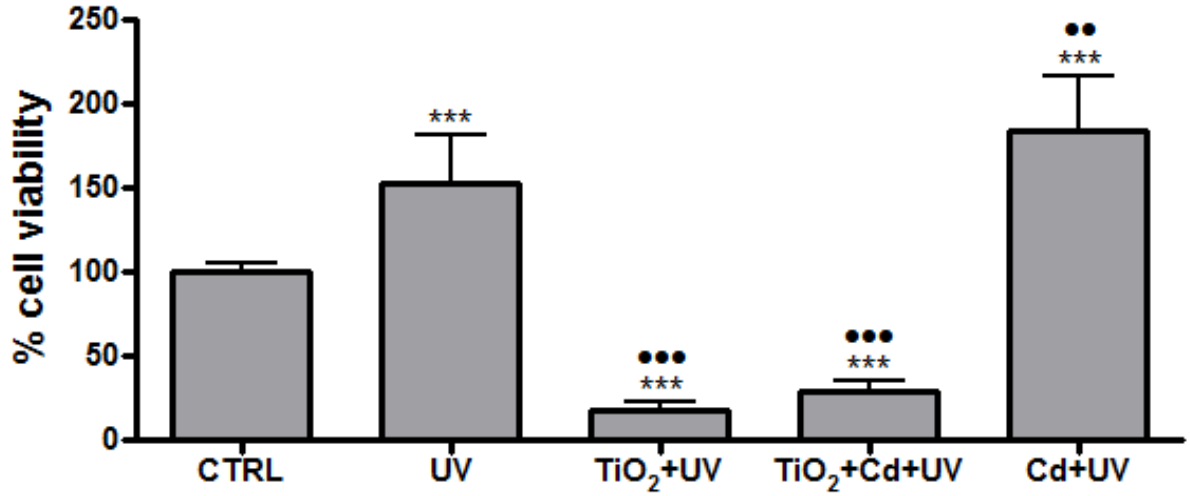


A) TiO_2-NPs 1 µg/ml



B) TiO_2-NPs 1000 µg/ml

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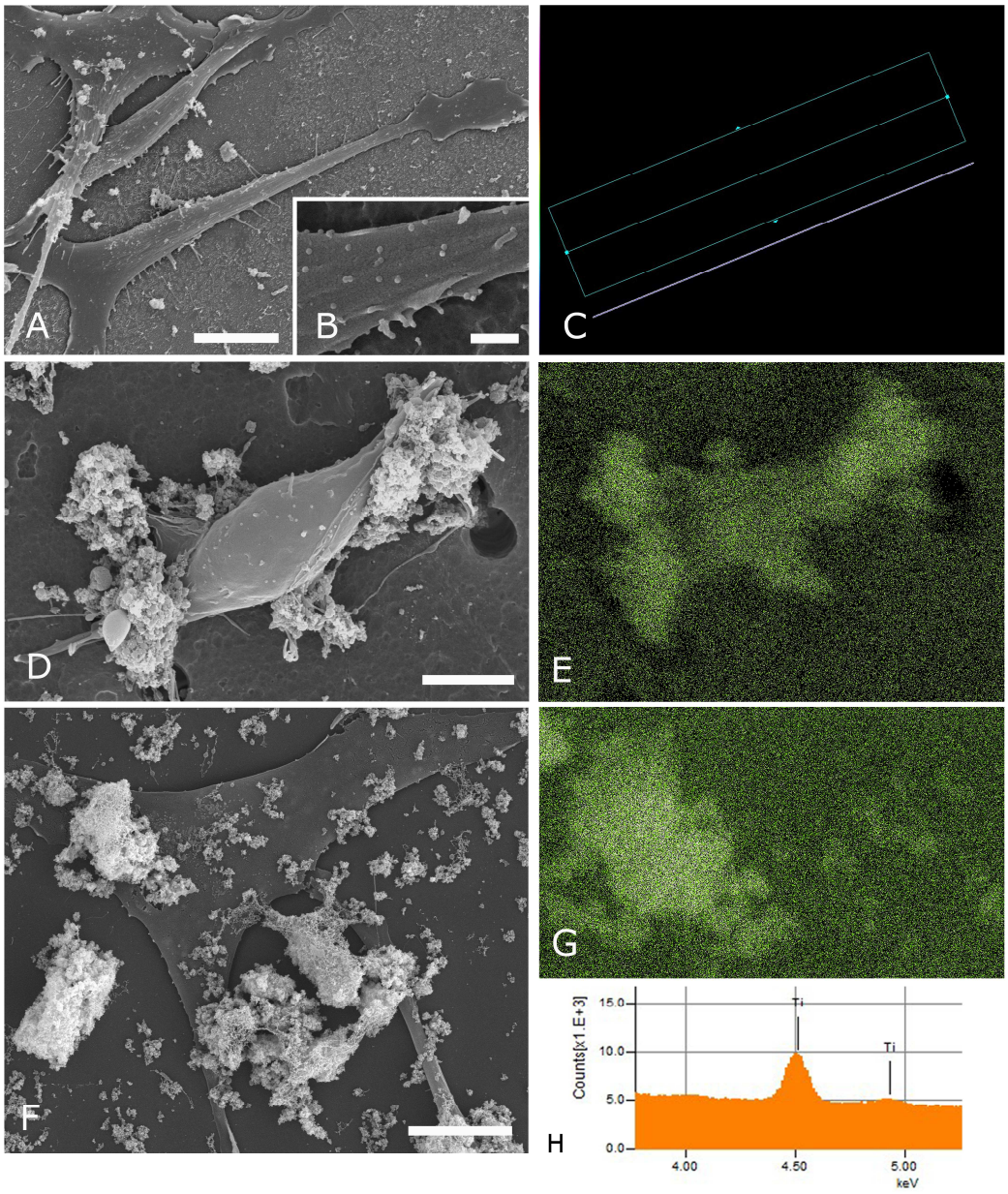
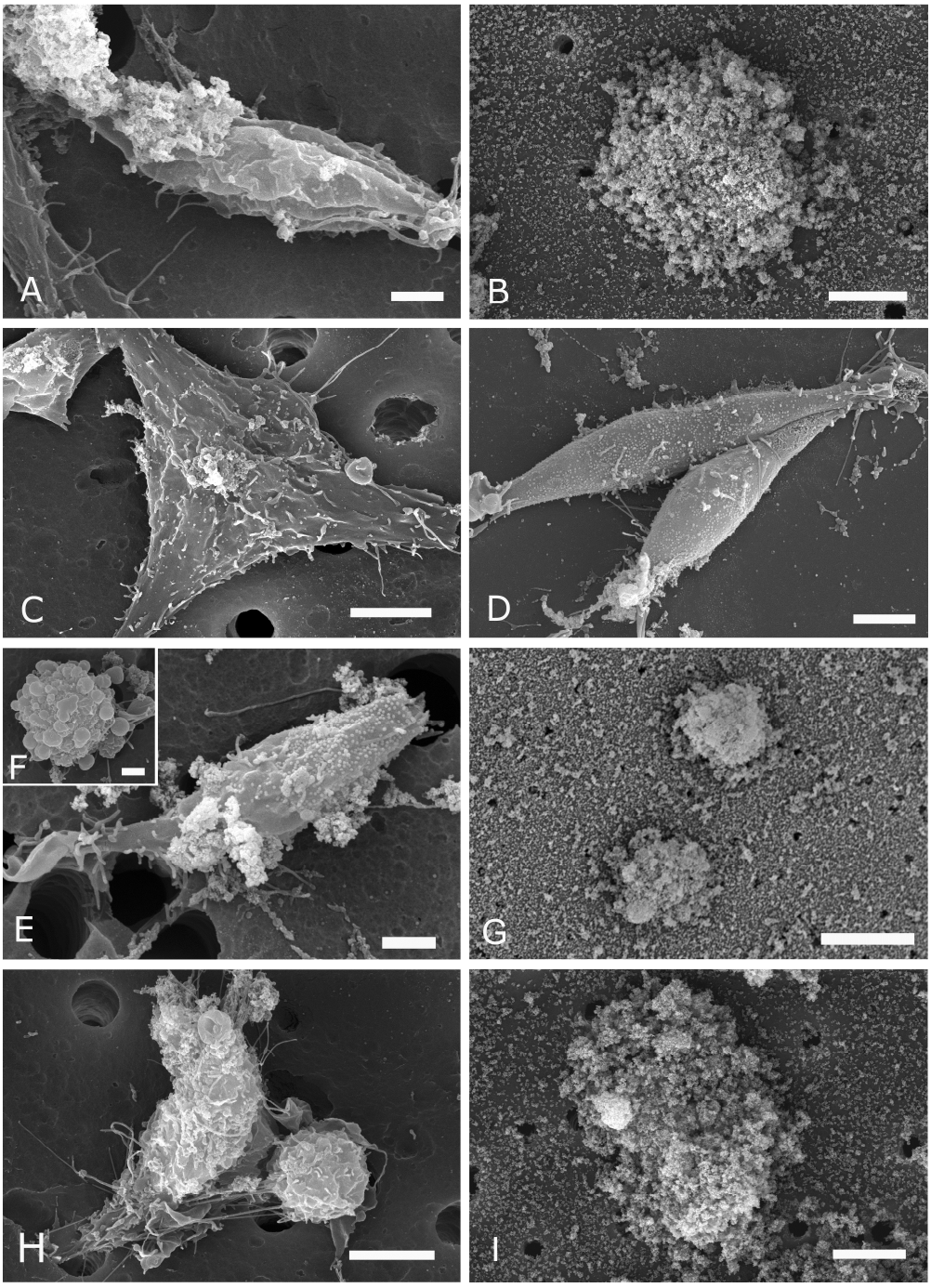
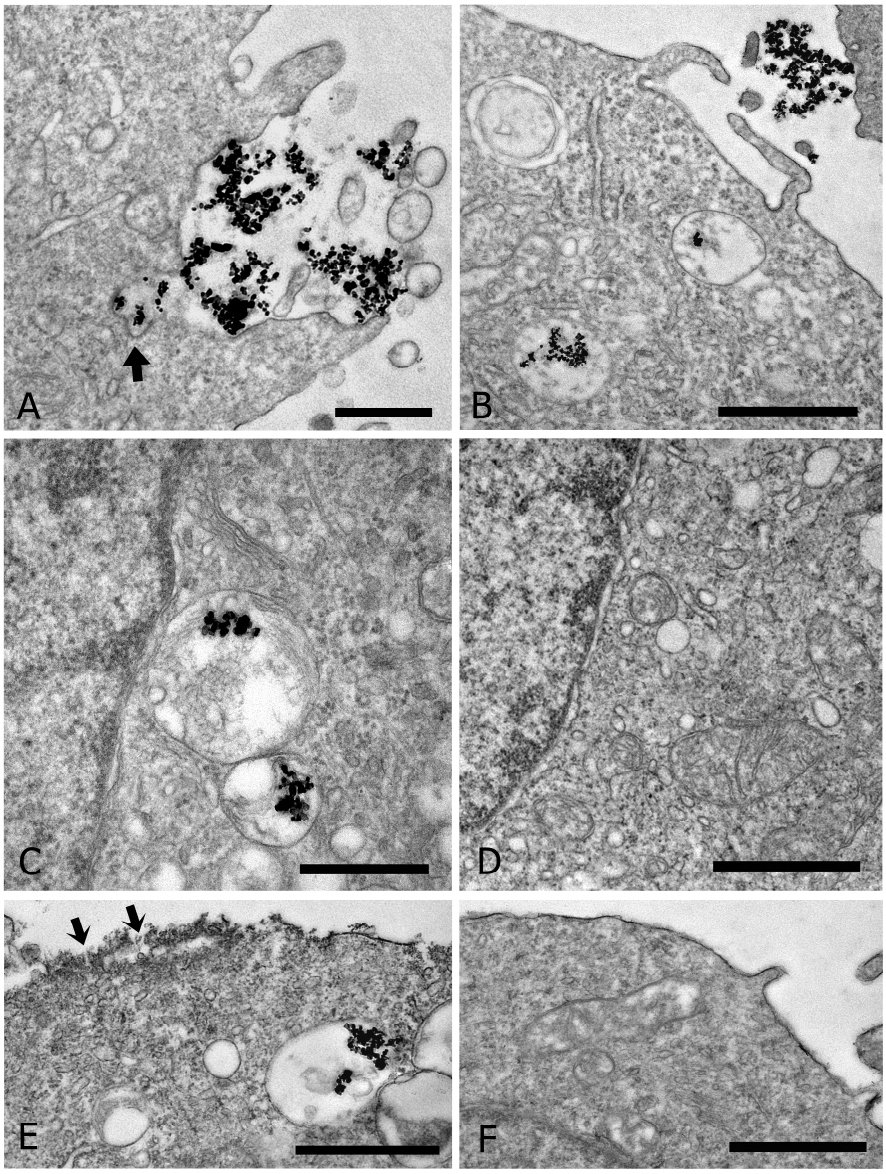
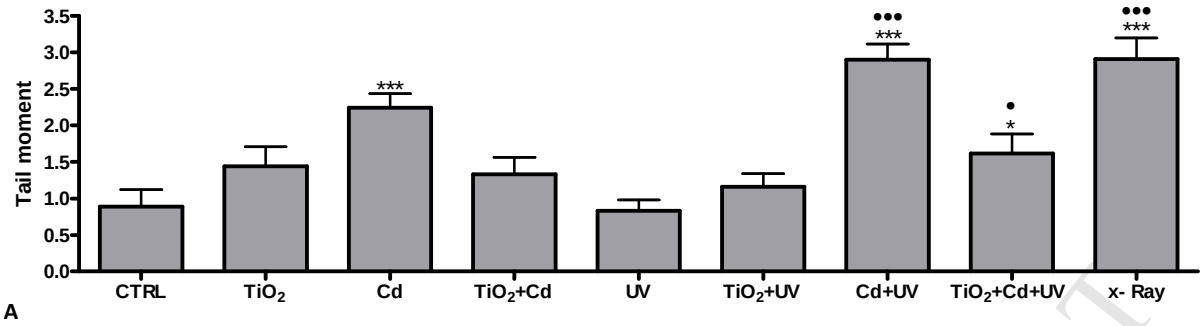
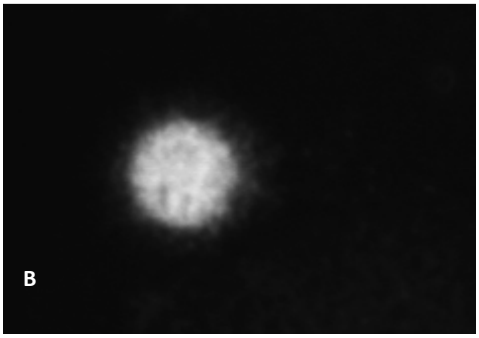

A
B
C
D
E
F
G
H
Counts[x1 E+3]
15.0
10.0
5.0
0.0
Ti
Ti
4.00
4.50
5.00
keV

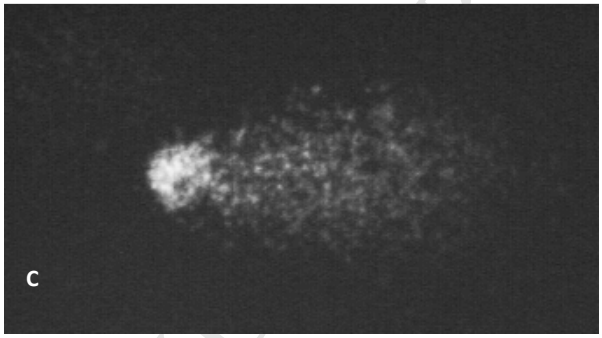

A
B
C
D
F
E
G
H
I


A
B
C
D
E
F



A





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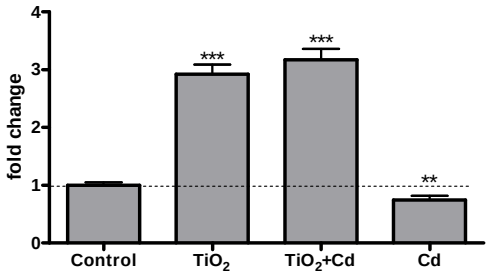
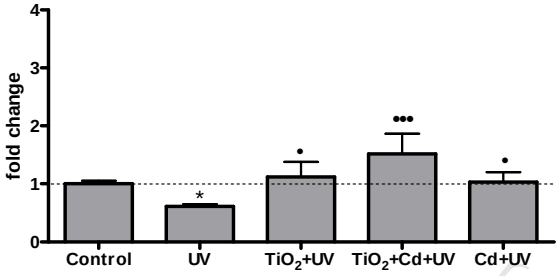
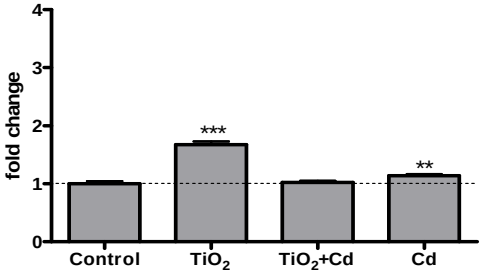


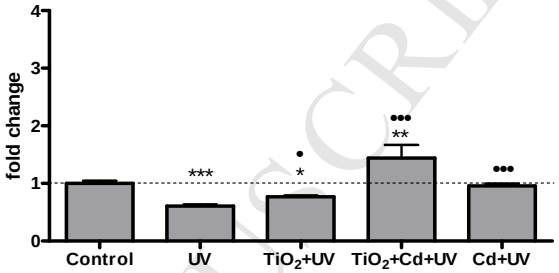
A.



B.



C.



D.

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HIGHLIGHTS

- TiO_2-NPs were internalized by DLEC cells and the addition of Cd did not interfere with the internalization process.
- Photoactivation of TiO_2-NPs and sorption with $CdCl_2$ (Cd) contribute to induce cellular toxicity.
- DNA damage was revealed in DLEC cells treated with photoactivated TiO_2-NPs in presence of Cd.
- DLEC cells do not require caspase-3 for apoptosis induced by TiO_2-NPs under UV and Cd exposure.
- IL-8 and TGF-β transcripts were up-regulated after TiO_2-NPs treatment and TGF-β level also increased after TiO_2-NPs+UV+Cd exposure.

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